The role of Pseudomonas syringae effector AvrPto in promoting bacterial virulence and eliciting plant immunity

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THE ROLE OF *PSEUDOMONAS SYRINGAE* EFFECTOR AVRPTO IN PROMOTING BACTERIAL VIRULENCE AND ELICITING PLANT IMMUNITY

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The use of the type III secretion system (T3SS) to deliver a suite of effector proteins into the host cell cytoplasm is a common virulence strategy employed by many Gram-negative bacteria during host infection. Studies of bacterial effectors' functions inside the host cell reveal that many effectors have evolved to utilize and/or interfere with the eukaryotic host machinery to promote bacterial virulence. *Pseudomonas syringae* pv. *tomato* effector AvrPto is one of the best-characterized plant bacterial effectors. AvrPto is a modular protein with two distinct virulence determinants: the CD loop in the core structure and the phosphorylated C-terminal domain (CTD). The CD loop structure is required for the ability of AvrPto to interfere with pattern recognition receptor complexes to suppress host's pathogen associated molecular patterns (PAMPs)-triggered immunity (PTI). This domain is monitored by the tomato resistance protein complex Pto/Prf via the direct binding of Pto to AvrPto. The CTD of AvrPto is phosphorylated by a yet unknown host kinase, whose activity is conserved in many plant species. I describe here that the phosphorylated CTD contributes additively to AvrPto virulence together with the CD loop. The mechanism by which the CTD promotes bacterial virulence is unknown; however, it is different from that of the CD loop. The CTD is specifically recognized by a novel resistance only found in *Nicotiana* species; therefore, two distinctive host recognition mechanisms have evolved to monitor the two domains of AvrPto. These two virulence

domains are functionally conserved in certain AvrPto homologs from other *P. syringae* pathovars, indicating that they might target host processes that are conserved in many plant species. Detailed characterization of AvrPto homologs has revealed a possible advantage of modularity for bacterial effector, which is the ability to avoid host recognition but still retain partial virulence.

I investigated the host kinase activity, Avk for AvrPto kinase, that phosphorylates the AvrPto CTD. Avk activity is enhanced upon treatments with PAMPs, and T3SS effectors could suppress this induction. Using functional protein microarrays, I screened for plant kinases that could phosphorylate the CTD *in vitro* and identified calcium-dependent protein kinases (CDPKs) as potential Avks. Further characterization of Avk activity revealed that it is dependent on Ca^{2+}, which supports the role of CDPKs as Avks. Thus, AvrPto has evolved to be a suitable substrate of a conserved plant kinase family for its activation; however, more work needs to be directed toward the investigation of whether or not a member of this kinase family are the virulence target of the AvrPto CTD.

BIOGRAPHICAL SKETCH

Hanh Phuong Nguyen was born and raised in Ho Chi Minh City, Vietnam. After completing Le Hong Phong High School for the Gifted in 2002, she moved to the United States to continue her education. Hanh attended Truman State University in Kirksville, MO and graduated with a Bachelor of Science concentrating in Biology (*Summa Cum Laude*) in 2005. It was at Truman State that Hanh discovered her interest for plant biology, in particular molecular biology and genetics. She went on to do a PhD at the Department of Plant Biology, Cornell University. There, she joined the laboratory of Prof. Gregory Martin in 2006 to study the molecular interactions between plants and pathogens. Besides doing research, Hanh is actively involved in many outreach activities and event planning. She also enjoys travelling, music, cooking, and being outdoors.

For my mother, who has taught me the love for knowledge

(Cam on me da day cho con tinh yeu kien thuc.)

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CHAPTER 1

INTRODUCTION

The well-known phenomenon that plants are resistant to most pathogens has become better understood in recent years due, in part, to the identification of pattern recognition receptors (PRRs) that allow plants to detect certain conserved and usually indispensible features of microbes (pathogen- or microbial- associated molecular patterns, PAMPs or MAMPs) (Boller and Felix, 2009). This recognition triggers a cascade of defense responses, termed PAMP-triggered immunity (PTI), that protect plants against potential pathogenic microorganisms (Asai et al., 2002; Boller and Felix, 2009). It is, therefore, logical to hypothesize that, successful pathogens have evolved to avoid or suppress PTI.

Bacterial pathogens have, in fact, evolved sophisticated strategies to subvert the host surveillance system for their own benefit. One example is the use of the type III secretion system (T3SS), a molecular 'syringe' extending through the plant cell wall and plasma membrane, by many Gram-negative bacteria to inject a suite of effectors into the plant cell during the infection process (Jin and He, 2001; Alfano and Collmer, 2004). Advances in the functional characterization of many effectors have revealed that although they are different in their enzymatic activities and tertiary structures, their effects are similar, which involve disrupting PTI (Chisholm et al., 2006; Cunnac et al., 2009). Plants, in turn, have evolved resistance genes to detect the presence of these effectors to trigger a much stronger response, termed effector-triggered immunity (ETI), which is typically associated with the hypersensitive response (HR) or programmed cell death (PCD) to inhibit bacterial proliferation. The 'arms race' goes on with the bacteria finding ways to counter plant defense strategies,

and vice versa. The outcome of this dynamic and multilayered interaction between the host immune system surveillance and the pathogen strategies to overcome it determines the survival of each organism.

The model pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), the causative agent of bacterial speck disease in tomato and necrosis symptoms in the model plant Arabidopsis, is a widely accepted model in the study of bacterial pathogenesis. The pathogen translocates approximately 30 effectors via the T3SS into the host cell during infection (Alfano and Collmer, 2004). *Pst* with a disrupted T3SS grows poorly and cannot cause disease symptoms, indicating the importance of the effector repertoire (Roine et al., 1997). However, due to functional redundancy, deletion of most individual effectors does not have an effect on bacterial fitness (Kvitko et al., 2009). AvrPto is one of the few effectors that have been shown to contribute significantly to bacterial virulence in the host plant (Lin and Martin, 2005). Together with AvrPtoB, another effector from *Pst*, AvrPto can suppress PTI in all assays tested while other effectors only suppressed one or two PTI responses (Cunnac et al., 2009). Characterization of AvrPto functions and the host surveillance systems monitoring its activity have revealed many steps in the co-evolutionary 'arms race' between plants and pathogens.

AvrPto is a small (18 kDa) hydrophilic protein with a four α-helix bundle at the core of its structure (Ronald et al., 1992; Wulf et al., 2004). It was first identified based on its role in eliciting 'gene-for-gene' resistance in tomato plants expressing the resistance genes *Pto,* encoding a Ser/Thr kinase, and *Prf,* encoding a typical nucleotide-binding leucine rich repeat protein (NB-LRR) (Martin et al., 1993; Salmeron et al., 1996). Since its identification almost 20 years ago, AvrPto has been studied extensively. About 30% of its residues have been substituted for functional characterization (Shan et al., 2000a; Chang et al., 2001; Anderson et al., 2006;

Pascuzzi, 2006). Findings from these studies revealed three functional domains of AvrPto with the first being an N-terminal myristoylation motif that is processed by a host myristoyl-transferase for plasma membrane targeting (Shan et al., 2000b). Proper membrane localization is essential for AvrPto function both as a virulence and an avirulence factor (Shan et al., 2000b; Thara et al., 2004). The second domain of AvrPto lies within the omega loop, packed between helices C and D (residues 82 to 102), from here on referred to as the CD loop. The CD loop has been indicated to mediate protein-protein interactions (Wulf et al., 2004). Beside N-terminal myristoylation, AvrPto undergoes an additional host-mediated post-translational modification upon being delivered into the host cell: phosphorylation on Ser residues at the carboxyl-terminus by a yet unknown kinase(s) (Anderson et al., 2006). The phosphorylated C-terminus represents the third functional domain of AvrPto. The CD loop and the C-terminal domain both contribute to AvrPto virulence and are monitored by different host recognition mechanisms (Yeam et al., 2010).

The CD loop targets PAMP receptor complex to suppress PTI and is recognized by Pto/Prf in tomato

As mentioned earlier, many residues of AvrPto have been substituted for functional analysis; however, most substitutions did not affect its function. Only a few substitutions, mostly within the CD loop, showed an effect on AvrPto ability to enhance bacterial growth in susceptible plants and to trigger Pto/Prf-mediated recognition in resistant plants (Shan et al., 2000a; Pascuzzi, 2006). The NMR solution structure of AvrPto indicates that these changes would alter the CD loop structure, suggesting that they might disrupt the interaction between AvrPto and its host target(s) (Wulf et al., 2004). In an early study, Arabidopsis plants overexpressing AvrPto

allowed the T3SS deficient mutant of *Pst* to grow almost to the same level as the wild-type bacteria (Hauck et al., 2003). This remarkable result suggests that AvrPto, when present in a large amount, sufficiently suppresses PTI to allow significant growth of the T3SS mutant. In addition, AvrPto was shown to suppress the cell wall-based defense response of callose deposition induced by the T3SS mutant. Another study showed that AvrPto could suppress PTI-associated reduced vascular staining in *Nicotiana benthamiana* (Oh and Collmer, 2005). Using an Arabidopsis protoplast system, it was demonstrated that AvrPto action is likely to be at the early stage of the PTI pathways, upstream of the mitogen-activated protein kinase (MAPK) signaling pathway, possibly at the level of PAMP perception (He et al., 2006). However, the molecular and biochemical mechanisms underlying these observations remained elusive.

In tomato plants carrying the resistance protein complex Pto/Prf, AvrPto is recognized by the direct interaction between AvrPto and Pto, which leads to ETI (Tang et al., 1996). Prf is required for this recognition to occur. The two-component host resistance, as in the case of Pto/Prf, seems to be a common feature in host plant recognition of effector (Collier and Moffett, 2009). Similar recognition systems have been described in the literature, including RIN4/RPM1-mediated recognition of AvrRpm1 and AvrB; RIN4/RPS2-AvrRpt2; and RPS5/PBS1-AvrPphB (Jones and Dangl, 2006). These systems also involve a host R protein, often an NB-LRR, acting in concert with another host protein to detect the presence of a bacterial effector and trigger ETI. The ‘guard hypothesis’ was proposed to explain the mechanism by which the two host components mediate effector recognition. In this hypothesis, the resistance protein ‘guards’ one of the virulence targets of the effector, the ‘guardee’, and triggers defense response when the effector modifies its target (Jones and Dangl, 2006). This hypothesis was developed to explain Pto/Prf-mediated recognition of

AvrPto, in which Pto would be the virulence target of AvrPto guarded by Prf. A piece of evidence to support this hypothesis is the fact that changes made in residues abolishing the AvrPto-Pto interaction also reduce AvrPto virulence. However, most efforts to obtain evidence supporting this hypothesis have not been conclusive, not only for AvrPto-Pto/Prf interaction but also for other systems (Belkhadir et al., 2004; Pascuzzi, 2006). The interaction of AvrPto with Pto does not increase *Pst* virulence in the absence of *Prf* (Pascuzzi, 2006). This result indicates that if Pto is in fact a virulence target, there are other important virulence targets too. In addition, besides its significant role in ETI, the role of Pto in other defense pathways (i.e. PTI) is unclear. Alternatively, Pto could play a prominent role in mediating AvrPto recognition but not in promoting AvrPto virulence; therefore, the 'guard hypothesis' might not explain Pto/Prf-mediated recognition of AvrPto.

It was thought that AvrPto activates Pto kinase activity and subsequently initiates resistance, which requires the action of Prf (Pedley and Martin, 2003). However, evidence for this hypothesis has not been forthcoming. A recent structural study showed the crystal structure of AvrPto-Pto complex revealing the interaction of the two proteins, which involves two interfaces (Xing et al., 2007). The first interface is primarily mediated through the interaction between residues at the ends of helices C and D of AvrPto and one Pto loop. The second interface involves the CD loop interacting with the P+1 loop of Pto, which confirms the results from previous mutagenesis studies. This study also showed that AvrPto acts as an inhibitor of Pto kinase *in vitro* in contrast to the previous belief. Residues within the CD loop are important for AvrPto kinase inhibition activity (Xing et al., 2007). Interestingly, although inhibition of Pto kinase activity is not required for AvrPto to trigger Pto/Prf-dependent resistance, AvrPto kinase inhibition activity is important for its virulence

function. This observation led Xing and colleagues to postulate that other Pto-like kinases or Pto-like receptor kinases might be the virulence targets of AvrPto.

There are a few receptor-like kinases (RLKs) involved in PTI characterized to date, including FLS2, the receptor of bacterial flagellin (Gomez-Gomez and Boller, 2000); EFR, the receptor of elongation factor Tu (EF-Tu) (Zipfel et al., 2006), and CERK1, a LysM extra-cellular domain receptor of fungal chitin and an unknown bacterial PAMP (Miya et al., 2007; Gimenez-Ibanez et al., 2009). AvrPto indeed binds the kinase domains of FLS2 and EFR and inhibits their kinase activity in a dosage-dependent manner, consistent with its plasma membrane localization and other activities (Xiang et al., 2008). These findings suggest that Pto may act as a 'decoy' of AvrPto virulence targets, the host RLKs. In fact, there seems to be a competition between Pto and FLS2 for AvrPto binding since overexpressing either FLS2 or Pto could partially relieve AvrPto-mediated suppression of MAPK signaling pathway (He et al., 2006). Pto and Prf are suggested to be in a stable complex *in vivo*, in which Pto acts as a switch for the hypersensitive response (Mucyn et al., 2006). The structural study suggests that the binding of AvrPto would 'unlock' and induce conformational changes in Pto, which alters the way it interacts with Prf and thereby activates Prf (Xing et al., 2007).

Plant RLKs form a monophyletic group indicating that AvrPto might bind and inhibit the kinase activity of other RLKs involved in PTI in addition to FLS2 and EFR (Shiu and Bleecker, 2001). In fact, AvrPto could interact with and suppress the kinase activity of an uncharacterized RLK, At2g23200 (Xiang et al., 2008). Although the exact amount of AvrPto delivered into the plant cell during infection is still unknown, it may seem surprising that there would be enough AvrPto to bind to and suppress the kinase activity of all the induced PRRs. However, it is probably important to consider that AvrPto is likely to be localized at a high concentration within a microdomain of

the plasma membrane where the infection occurs. At this point of contact, even a small amount of AvrPto may therefore be sufficient to disrupt the receptor kinases and effectively interfere with PTI signaling.

A later finding that AvrPto binds to BAK1, the common co-receptor of many PRRs, has raised an alternative explanation for AvrPto mode of action (Chinchilla et al., 2007; Shan et al., 2008). By targeting BAK1, AvrPto could disrupt many PRR pathways. AvrPto was shown to bind to BAK1 with a higher affinity than to FLS2 and to disrupt the receptor complex and inhibit downstream PTI signaling. Despite certain inconsistencies, the interpretation of these two studies, may not be mutually exclusive. For example, AvrPto may bind and inhibit kinase activity of FLS2 while also targeting and disrupting BAK1-related complexes. Both studies provide the first evidence for the biochemical function of the CD loop of AvrPto, which is targeting PAMPs-receptors. Interestingly, although the perception of many PAMPs is disrupted in *bak1* mutant plants, the perception of many more PAMPs are affected by ectopic expression of AvrPto (Shan et al., 2008). Therefore, AvrPto could also target other RLK complexes that are BAK1-independent. Another recent study shows that the CD loop of AvrPto suppresses the expression of certain miRNAs that are induced during PTI. This suppression may be a downstream effect of AvrPto inhibition of PRR activities or could feasibly represent another activity of the effector (Navarro et al., 2008).

AvrPto is usually ectopically over-expressed in PTI-inhibition assays with one exception is the assay used to demonstrate AvrPto-mediated disruption of FLS2-BAK1 association, which was shown also in the context of a natural infection (He et al., 2006; Shan et al., 2008). The scope of AvrPto activity when being delivered at its native level from *Pst* needs to be further evaluated. Could a single effector suppress multiple PTI processes? There are other effectors from *Pst* that could suppress PTI, including AvrPtoB, which shares some targets with AvrPto. Interestingly, the

virulence activities of AvrPto and AvrPtoB during a natural infection process have been shown to be additive not redundant, indicating they might target some different PTI targets (Lin and Martin, 2005). Although the functions and targets of other effectors are still largely unknown, it is likely that many of them could disrupt similar host processes as those demonstrated for AvrPto (and AvrPtoB) in assays using ectopic expression of the effector. In fact, while the reduction in growth of *Pst* lacking AvrPto is quite subtle, though significant, as compared to the wild-type *Pst*, overexpression of AvrPto in plants restores the growth of the T3SS mutant almost to the wild-type level (Hauck et al., 2003; Lin and Martin, 2005). This indicates that simultaneous actions of multiple effectors are probably needed for *Pst* to sufficiently suppress various host PTI pathways in a compatible interaction. Moreover, as in most organisms, functional redundancy is likely an evolutionary advantage for bacterial pathogen, especially in the 'arms race' where the host defense mechanism can recognize a single effector to trigger immune response.

The phosphorylated C-terminal domain (CTD) promotes virulence in tomato and is recognized by Rpa in tobacco

Upon delivery into the plant cell, AvrPto is phosphorylated by a host kinase activity that is Pto/Prf-independent and is conserved in many plant species, termed Avk (for AvrPto kinase) (Anderson et al., 2006). Three serine residues at the carboxyl terminal, Ser 147, 149, and 153, were identified as *in vitro* phosphorylation sites. Of these, S149, was confirmed as an *in vivo* site using mass spectrometry. Alanine substitutions at S147, S149, or S153 reduce AvrPto virulence activity in susceptible tomato plants and also subtly affect AvrPto recognition in tomato plants carrying Pto/Prf (Anderson et al., 2006). Our recent study provides evidence that the CTD and the CD loop contribute additively to AvrPto virulence (Yeam et al., 2010). In contrast

to the CD loop function, CTD substitutions (S147A and S149A) do not abolish AvrPto ability to suppress MAPK activity induced by PAMPs, namely flg22, chitin, or PGN. This indicates that the CTD does not target RLK complexes, at least not those that are involved in detecting the PAMPs tested. Therefore, the CTD promotes virulence via a distinct mechanism from that of the CD loop (Yeam et al., 2010).

As an interesting corollary to Pto/Prf-mediated recognition of the CD loop in tomato, the CTD is recognized by an uncharacterized recognition mechanism in cultivated tobacco (*N. tabacum* W38) (Shan et al., 2000b). Further characterization of this recognition showed that the hypothesized R gene (termed Rpa, for Recognition of phosphorylated AvrPto), monitors the phosphorylation status of S147 and S149 and not simply the structure of the C-terminal region (Yeam et al., 2010). Cultivated tobacco is a complex amphidiploid thought to be derived from a natural hybridization between two diploid species, *N. sylvestris* and *N. tomentosa*. These two wild species have different AvrPto-recognizing mechanisms: *N. sylvestris* recognizes the CTD (Rpa) while *N. tomentosa* has Pto-like recognition of the CD loop. Interestingly, Rpa from *N. sylvestris* is preferentially retained in *N. tabacum.* Therefore, it was speculated that Pto-like recognition might have been lost in *N. tabacum* since Rpa provides sufficient protection against AvrPto (Yeam et al., 2010). When we further examined AvrPto recognition in wild *Nicotiana* and *Solanum* species, *Rpa* was found only in species within the *Nicotiana* genus while Pto-like recognition was detected in both *Nicotiana* and *Solanum*. However, we never observed both recognition capabilities together in one accession, suggesting a potential fitness cost for them. In addition, only Pto-like recognition is observed in *Capsicum* and *Petunia* species (I. Yeam and G. B. Martin, unpublished data). Therefore, it seems that *Rpa* has arisen independently within *Nicotiana* species. The cloning of *Rpa* will shed light on the differences and similarities between the two recognition mechanisms.

Host-mediated phosphorylation is not unique to AvrPto. Other effectors are known to be phosphorylated by unknown host kinase activities including *P. syringae* effectors AvrPtoB and AvrB, and *Rhizobium* sp. NGR234 effectors NopL and NopP (Bartsev et al., 2003; Skorpil et al., 2005; Desveaux et al., 2007; Xiao et al., 2007a). However, AvrPto and AvrPtoB are the only effectors that have their *in vivo* and *in vitro* phosphorylation sites mapped and demonstrated experimentally to be important for virulence and avirulence activities. In the case of AvrB, its phosphorylation in the presence of plant extracts is dependent on the conserved nucleotide-binding residues, which are important for its virulence activity, RIN4 binding and subsequently triggering RPM1-dependent resistance (Desveaux et al., 2007). Thus, the phosphorylation status of AvrB is correlated with its virulence and avirulence activities. Besides a suggestion that MAPKs may phosphorylate NopP, to date no host kinase has been identified to directly phosphorylate any effector, nor has any molecular function been demonstrated for the phosphorylated residues (Skorpil et al., 2005). It seems, however, that there is a class of effectors that require phosphorylation by host kinases for activation.

Many kinase-encoding genes are transcriptionally upregulated during PTI (Navarro et al., 2004; Zipfel et al., 2004). AvrPto, and perhaps other effectors, might take advantage of this enhanced kinase activity to become phosphorylated. It is also possible that the AvrPto CTD targets these kinases as a mimic of their substrates as part of its virulence function. The type IV effector, CagA from *Helicobacter pylori* which is secreted directly into gastric epithelial cells also localizes to the plasma membrane and is phosphorylated on tyrosine residues at its C-terminus by kinases in the Src family (Selbach et al., 2002). Phosphorylation of CagA by Src, in turn, deactivates c-Src via a negative feedback loop, which promotes the rearrangement of the host cell's actin cytoskeleton resulting in the gastric disease-associated

'hummingbird' phenotype (Selbach et al., 2003). It would be interesting to test whether or not the AvrPto CTD also inhibits Avk signaling pathway(s) in a similar manner to CagA. The eventual identification and characterization of Avk should elucidate the biochemical and molecular virulence functions of this domain.

The advantage of modularity for bacterial effectors in their interactions with the host immune system

The AvrPto-Pto/Rpa interactions nicely demonstrate that in order to ensure durable recognition of an effector protein, the host surveillance system has evolved to recognize the precise domains that are important for effector virulence function. Pto and Rpa target the two essential virulence domains of AvrPto, the CD loop and the CTD, respectively, to trigger ETI. Although we have not yet been able to demonstrate virulence activity of either the CTD or the CD loop in *Nicotiana* species due to the lack of an accession that does not express Pto or Rpa, it is reasonable to hypothesize that Rpa and Pto have arisen in *Nicotiana* species to detect these domains of AvrPto. It is surprising that while the CTD has virulence activity in tomato, Rpa has not been detected in any tomato species tested. However, there is evidence that the CTD subtly impacts Pto/Prf-mediated recognition of the CD loop and so that recognition system may also have evolved to counter the CTD function (Anderson et al., 2006).

Some effectors have evolved different strategies to escape or counter against host recognition such as pathoadaptation[1] and HR suppression. The interaction between HopZ1 family and its host recognition nicely exemplifies the co-evolutionary arms race between a bacterial effector and the host, in which pathodaptation serves as an important mechanism (Ma et al., 2006). Cysteine protease activity of effectors

[1] Pathoadaptation is any change that occurs when a bacterial pathogen adapted to a new pathogenic niche, which is usually achieved through minor changes in a preexisting gene (Ma et al. 2006).

belonging to the HopZ1 family is required for their virulence activity and is closely monitored by the corresponding plant resistance genes. Due to strong selection pressure from the host, the ancestral form HopZ1a has been replaced by mutational derivatives to avoid recognition; however, its cysteine protease activity is still maintained (Zhou et al., 2009). Although the mechanism by which HopZ1 has evolved to escape host recognition while maintaining its virulence activity is unknown, pathoadaptation is an important mechanism that bacterial effectors employ under strong selection pressure from the host.

At least 9 T3SS effectors could suppress HR in *planta* and PCD in yeast when expressed under a strong promoter (Jamir et al., 2004). However, the ability to suppress immunity to restore pathogenicity in a natural infection process has only been demonstrated for two effectors: AvrRpt2 and AvrPtoB (Kim et al., 2005; Rosebrock et al., 2007). AvrRpt2 could suppress RPM1-mediated immunity in Arabidopsis by cleaving the RPM1-interacting partner RIN4 while AvrPtoB uses its C-terminal E3 ligase domain to degrade the resistance protein Fen, which interacts with its N-terminal region. Although it is too early to draw any conclusions about the relationship of AvrPto with its host recognition mechanism, its modularity with multiple virulence domains might be an evolutionary advantage (Yeam et al., 2010). Our investigation of AvrPto homologs provides further indication that modularity might be a bacterial strategy to lessen the cost of escaping host recognition by retaining partial virulence functions (Nguyen et al. 2010).

AvrPto-like sequences are detected in many pathovars implying that they might contribute to virulence in these pathovars (Lin and Martin, 2007). By cloning and characterizing the most divergent homologs, we found that despite many polymorphisms observed throughout the protein sequences, the key residues essential for AvrPto virulence are conserved in many homologs, suggesting selection pressure

to retain these residues (Nguyen et al., 2010). We showed that these AvrPto homologs could indeed promote bacterial virulence via the same mechanism as AvrPto$_{tomato}$ indicating that they might target similar host processes in their respective host plants. AvrPto homologs with the conserved CD loop and/or CTD are recognized by Pto and Rpa, respectively. Interestingly, one of these homologs, AvrPto$_{myricae}$ does not have a conserved CD loop and thereby does not trigger Pto/Prf-dependent resistance, yet it has the two conserved serines at positions 147 and 149. This homolog retains CTD-dependent virulence in susceptible tomato plants and is recognized by Rpa in tobacco.

A recent study examining *avrPto* genes in multiple *Pst* race 1 strains found in tomato fields in California, where the Pto locus has been widely introgressed from the wild species *S. pimpinellifolium* into tomato cultivars, identified a novel AvrPto allele that has a key residue within the CD loop altered (Kunkeaw et al., 2010). This race 1 strain, as expected, no longer triggers Pto/Prf-mediated resistance. The CTD of this AvrPto, on the contrary, remains intact. Growth assay indicates that this AvrPto allele could still enhance *Pst* virulence on susceptible tomato, suggesting that the CTD could be functional (Kunkeaw et al., 2010). Together with our results, this finding supports our hypothesis that multiple virulence domains might be a common feature in bacterial T3SS effectors. Indeed, there are examples of other modular T3SS effectors such as AvrPtoB and *Salmonella typhimurium* effector SptP. AvrPtoB has an N-terminal domain involved in suppressing PTI and a C-terminal E3 ligase domain suppressing ETI (Xiao et al., 2007). SptP has an N-terminal domain with high sequence similarity to a ribosyltransferase exo-enzyme and a C-terminal domain exhibiting tyrosine phosphatase activity (Kaniga et al., 2006).

This thesis describes my research, which has further demonstrated the complex interactions of AvrPto with its host virulence targets and corresponding recognition mechanisms, as well as the host components that phosphorylate its C-terminus.

Although some of my results have been incorporated into the previous sections, I summarize here the major findings that I have contributed during my dissertation research. First, my investigations provided molecular and systematic evidence for the additive effect of the two AvrPto conserved domains: the CD loop and the phosphorylated CTD to its overall virulence activity. I also distinguished the virulence-promoting mechanisms of the two domains: the CTD targets different host processes from the CD loop. Next, the cloning and detailed characterization of the most divergent AvrPto homologs from multiple strains of *P. syringae* showed that the two virulence domains are functionally conserved. While the host target of the CTD remains unknown, I discovered a strong connection among the host kinase(s) that phosphorylates the CTD upon AvrPto delivery into the plant cell, the virulence targets of this phosphorylated domain, and the host recognition that monitors its phosphorylation status. The eventual identification and characterization of these components will represent a novel class of molecular and biochemical functions of T3SS effectors in plants. I described in my last research chapter the identification and initial characterization of a family of host kinases that phosphorylate the CTD.

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CHAPTER 2

PHOSPHORYLATION OF *PSEUDOMONAS SYRINGAE* EFFECTOR AVRPTO IS REQUIRED FOR AN FLS2/BAK1-INDEPENDENT VIRULENCE ACTIVITY AND RECOGNITION BY TOBACCO[2]

Abstract

The type III effector protein AvrPto from *Pseudomonas syringae* pv. *tomato* is secreted into the plant cell where it promotes bacterial growth and enhances symptoms of speck disease on susceptible tomato plants. The virulence activity of AvrPto is due, in part, to its interaction with components of host pattern recognition receptor complexes which disrupts PAMP-triggered immunity. This disruption mechanism requires a structural element of the AvrPto protein, the CD loop, which is also required for triggering the Pto/Prf-mediated resistance in tomato. We have shown previously that the carboxyl-terminal domain (CTD) of AvrPto is phosphorylated and also contributes to bacterial virulence. Here we report that phosphorylation of the CTD on S147 and S149 promotes bacterial virulence in an FLS2/BAK1-independent manner, which is mechanistically distinct from the CD loop. In a striking corollary with Pto recognition of the CD loop in tomato, the tobacco species *Nicotiana sylvestris* and *Nicotiana tabacum* have a recognition mechanism that specifically detects the phosphorylation status of the CTD. Thus closely related species in the Solanaceae family have evolved distinct recognition strategies to monitor the same type III effector.

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[2] Adapted from I. Yeam, **H. P. Nguyen***, and G. B. Martin. 2010. Phosphorylation of *Pseudomonas syringae* effector AvrPto is required for an FLS2/BAK1-independent virulence activity and recognition to tobacco. The Plant Journal 61: 16-24. *co-first author

Introduction

Plants recognize the presence of attacking bacterial pathogens via the action of plasma membrane-localized pattern recognition receptors (PRRs) that detect specific microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) such as flagellin, lipopolysaccharide, cold shock protein, elongation factor TU (EF-Tu), and peptidoglycan (Abramovitch *et al.*, 2006; Boller and Felix, 2009). These recognition events trigger an early response, termed PAMP-triggered immunity (PTI), which limits bacterial growth. The PTI response includes increased activity of mitogen-activated protein kinases (MAPKs), the generation of reactive oxygen species, a calcium burst, expression of transcription factors and PRR genes, and callose deposition at the cell wall (Chisholm *et al.*, 2006; Boller and Felix, 2009). Bacterial pathogens overcome this first line of inducible defense by injecting into the plant cell, via the type III secretion system (T3SS), a repertoire of effector proteins that interfere with various steps of PTI (Chisholm *et al.*, 2006; Boller and Felix, 2009). Plants, in turn, have evolved a second inducible defense system that relies on the ability of their resistance (R) proteins to recognize the presence of specific effectors and to thereby trigger a strong immune response typically associated with localized host programmed cell death referred to as the hypersensitive response (HR). This effector-triggered immunity (ETI) presents a more robust layer of defense that restricts the proliferation of the pathogen. Recent studies show that certain pathogen effectors have successfully evolved to interdict ETI (Rosebrock *et al.*, 2007). These complex multi-layered interactions underlie the co-evolutionary 'arms race' between plants and pathogens.

The interaction between *Pseudomonas syringae* pv. *tomato* (*Pst)*, the causative agent of bacterial speck disease, and its host plant tomato serves as a model system for elucidating the molecular mechanisms underlying bacterial pathogenesis and plant immunity. The sequenced genome of *Pst* strain DC3000 revealed approximately 30

effectors that are injected into the plant cell via the T3SS during infection (Cunnac *et al.*, 2009). AvrPto, one of the best characterized of these effectors, is targeted to the plant plasma membrane via N-terminal myristylation where it promotes bacterial virulence (Ronald *et al.*, 1992; Chang *et al.*, 2000; Shan *et al.*, 2000b; Hauck *et al.*, 2003; Wulf *et al.*, 2004). Based on studies using Arabidopsis protoplasts it was hypothesized that AvrPto suppresses PTI by acting upstream of a MAPK cascade, possibly at the level of PAMP recognition (He *et al.*, 2006). Two recent reports support this hypothesis. In one, AvrPto was found to bind the kinase domain of FLS2 and EFR, PRRs that binds flagellin and EF-Tu, respectively, thereby inhibiting their activity and disrupting their ability to activate PTI (Xiang *et al.*, 2008). In the second, AvrPto was found to bind the kinase domain of BAK1 and to prevent the formation of the FLS2-BAK1 complex *in vivo* required for the activation of PTI (Shan *et al.*, 2008). Another recent study shows that AvrPto suppresses the expression of certain miRNAs that are induced during PTI; this suppression may be a downstream effect of AvrPto inhibition of PRR activities (Navarro *et al.*, 2008). *AvrPto*-like sequences are present in the genomes of many pathovars of *P. syringae* that infect a wide range of plant species (Ronald *et al.*, 1992; Lin and Martin, 2007). In tomato, AvrPto is recognized by the product of the *Pto* resistance gene, a member of a small gene family, encoding cytoplasmic serine/threonine protein kinases (Martin *et al.*, 1993). The Pto kinase physically interacts with AvrPto (and also another effector, AvrPtoB) in the plant cell, which triggers ETI (Scofield *et al.*, 1996; Tang *et al.*, 1996; Kim *et al.*, 2002). This recognition event requires the presence of the nucleotide-binding leucine-rich repeat protein Prf (Salmeron *et al.*, 1996; Mucyn *et al.*, 2006).

AvrPto is a small (18 kDa) hydrophilic protein (Ronald *et al.*, 1992) that is the target of several host-mediated post-translational modifications including myristylation and phosphorylation (Shan *et al.*, 2000b; Anderson *et al.*, 2006).

Previous extensive mutagenesis studies have identified functional domains important for AvrPto virulence activity and for mediating its recognition by the Pto kinase (Shan *et al.*, 2000b; Chang *et al.*, 2001; Wulf *et al.*, 2004; Xiang *et al.*, 2008). The structure of the AvrPto core (amino acids 31 - 124) has been solved by NMR and x-ray crystallography and consists of four a-helices with a 19-residue omega (Ω) loop lying between helices C and D (Wulf *et al.*, 2004; Xiang *et al.*, 2008). Mutations in the 'CD loop' decrease the virulence activity of AvrPto by disrupting its ability to interact with components of PRR/BAK1 complexes (Shan *et al.*, 2008; Xiang *et al.*, 2008). The CD loop forms one of two AvrPto contact surfaces that are recognized and bound by the Pto kinase (Xing *et al.*, 2007). Binding of AvrPto inhibits Pto kinase activity *in vitro* and leads to activation of Prf-mediated disease resistance (Mucyn *et al.*, 2006; Xing *et al.*, 2007). Substitutions altering the CD loop abolish the interaction with Pto, which, in turn, disrupts Pto/Prf-dependent ETI (Chang *et al.*, 2001; Wulf *et al.*, 2004; Xing *et al.*, 2007).

Another important structural element of AvrPto, the C-terminal domain (CTD; amino acids 146-164), is phosphorylated in the plant cell by a host kinase activity (Anderson *et al.*, 2006). This kinase activity is independent of either Pto or Prf and, in fact, is observed in diverse plant species including tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), and *Arabidopsis thaliana*. Three serine residues in the CTD of AvrPto, S147, S149 and S153, were implicated as phosphorylation sites by *in vitro* experiments and S147 was confirmed *in vivo* (Anderson *et al.*, 2006). Alanine substitutions at these phosphorylation sites reduce AvrPto virulence activity in tomato and weakly affect Pto-mediated recognition of the effector (Anderson *et al.*, 2006). In a striking parallel with CD loop recognition by Pto, the CTD is also recognized by a putative R protein in cultivated tobacco (*Nicotiana tabacum*) (Thilmony *et al.*, 1995; Shan *et al.*, 2000b). It was speculated that structural features of the CTD rather than

its phosphorylation status are recognized by the putative tobacco R protein (Anderson *et al.*, 2006).

Here we have examined the mechanism by which CTD phosphorylation promotes virulence activity of AvrPto and have investigated the role of this post-translational modification in the recognition of AvrPto by tobacco. Our results shed light on a mechanism by which a pathogen effector undermines the host immune response and the way in which certain plant species have evolved to counteract this pathogen manipulation.

Results

The CTD contributes to AvrPto virulence activity to a similar degree as the CD loop

Both the CD loop and the CTD of AvrPto are known to contribute to AvrPto virulence activity (Shan *et al.*, 2000a; Anderson *et al.*, 2006). In order to understand the relationship between these two elements, we developed a series of AvrPto variants altered in either one or both of these domains. Because phosphorylation of S153 is known to play less of a role in virulence we focused on S147 and S149 for these experiments (Anderson *et al.*, 2006). Each AvrPto variant was expressed in the *P. s.* pv. *tomato* DC3000D*avrPto*D*avrPtoB* strain and their effects on disease severity and bacterial populations were assessed on susceptible tomato Rio Grande-prf3 plants (Figure 2.1A and B). When expressed under its native *hrp* promoter and delivered by the T3SS, AvrPto enhanced the growth of DC3000Δ*avrPto*Δ*avrPtoB* about ten-fold more than a null mutant, AvrPto(G2A), that has a disrupted N-terminal myristylation motif (Shan *et al.*, 2000b). Either AvrPto(I96A), with an altered CD loop, or AvrPto(2xA; S147A/S149A) which disrupts CTD phosphorylation had reduced

virulence activity compared to AvrPto. When both the CD loop and the CTD were altered (AvrPto[I96A + 2xA]) the virulence activity of AvrPto was reduced to the level of AvrPto(G2A). Increased ethylene production was previously found to be associated with AvrPto virulence activity (Cohn and Martin, 2005) and so we also measured ethylene in susceptible plants infected with the same bacterial strains. Ethylene production was lower in plants inoculated with bacterial strains carrying AvrPto variants that affected either one of the domains and was reduced to the basal level with the strain carrying the CD loop/CTD-minus variant (Figure 2.1C). Protein expression and secretion of all AvrPto variants was similar to wild type AvrPto (Figure 2.1D and E). Based on these results, we conclude these two domains contribute additively to overall virulence activity of AvrPto.

The AvrPto CTD promotes virulence in tomato using a different mechanism than the CD loop

In *Arabidopsis* the enhanced virulence attributable to the AvrPto CD loop is associated with suppression of PAMP-induced MAPK activation (He *et al.*, 2006). To determine whether S147 and S149 play a role in this suppression in tomato, we assessed the effect of AvrPto variants on flg22-mediated MAPK activation using a protoplast system. We observed that AvrPto suppresses flg22-induced activation of the tomato MAPK, SlMPK3. Consistent with the previous results from *Arabidopsis* (He *et al.*, 2006), a substitution in the CD loop (I96A) greatly reduced the ability of AvrPto to suppress this activation (Figure 2.2A). However, alanine substitutions at both S147 and S149 had no effect on the ability of AvrPto to suppress MAPK activity. We also tested the ability of AvrPto to suppress MAPK activation by another PAMP, chitin (Shan *et al.*, 2008). Again, the CD loop and not the CTD was required to suppress MAPK activation resulting from host recognition of this PAMP (Figure 2.2B).

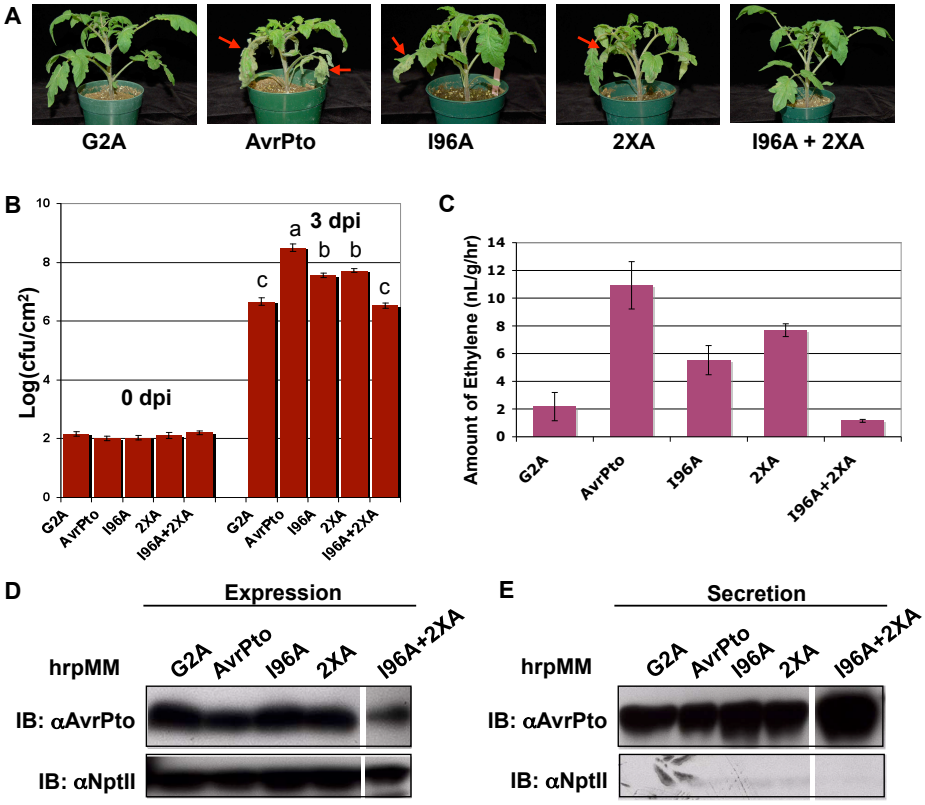

Figure 2.1. The two functional domains of AvrPto contribute additively to its virulence activity. (A) Disease symptoms observed on susceptible tomato RG-prf3 (*Pto/Pto prf/prf*) plants 5 days post-inoculation (dpi) with DC3000Δ*avrPto*Δ*avrPtoB* delivering AvrPto or the indicated AvrPto variants (10^4 cfu/mL). Red arrows point to enhanced disease symptoms compared to the G2A variant. **(B)** Bacterial populations in leaves of RG-prf3 at 0 and 3 dpi. Data are presented as colony-forming units (cfu) per square centimeter of leaf tissue. Letters above each bar represent groupings of statistical significance based on analysis of variance and comparisons for all pairs using Tukey-Kramer HSD ($P \leq 0.05$). Error bars indicate ± standard error (SE) (n=4). **(C)** Ethylene production in the same tomato plants as in (A) and (B) three days after bacterial inoculation. The amount of ethylene produced at earlier time points was close to zero (not shown). Error bars indicate ± SE (n=5). **(D)** Immunoblotting using an αAvrPto antibody was performed to detect expression of AvrPto proteins by *P. s.* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB*. **(E)** Immunoblotting using an αAvrPto antibody was performed to detect secretion of AvrPto from *P. s.* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB*.

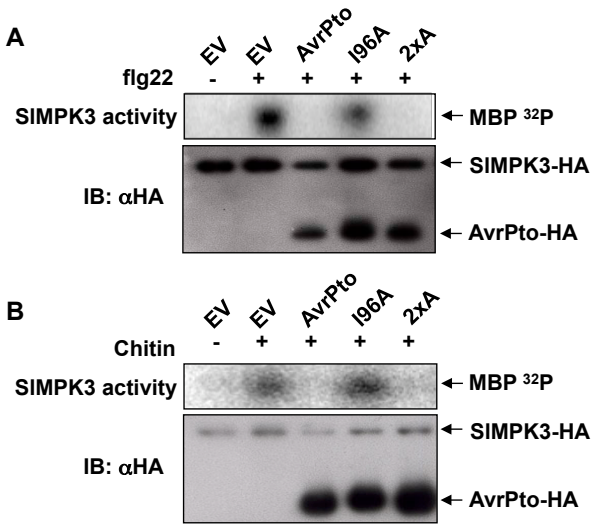

Figure 2.2. The S147 and S149 residues of AvrPto are not required to suppress activation of tomato MAPK (SlMPK3) by flg22 or by chitin. (A) HA-tagged AvrPto proteins were expressed with HA-tagged SlMPK3 in tomato RG-PtoS (*pto/pto Prf/Prf*) protoplasts. Transfected protoplasts were incubated for 6 hours and then treated with 100 nM flg22 for 10 minutes. EV indicates an empty vector control. **(B)** Tomato RG-*prf3* protoplasts transfected with HA-tagged SlMPK3 and the HA-tagged AvrPto constructs were incubated and then treated with 50 ug/mL chitin for 10 minutes. Anti-HA antibodies were used for the immunoprecipitation of SlMPK3 and AvrPto. An *in vitro* assay was used to detect SlMPK3 phosphorylation of myelin basic protein (MBP; upper panel). Lower panel shows a protein blot verifying expression of SlMPK3-HA and AvrPto-HA using anti-HA antibodies.

Phosphorylation of the CTD is required for its recognition by tobacco

Recognition of AvrPto by certain genotypes of either tomato or tobacco elicits resistance to *P. syringae*. In tomato, AvrPto is recognized via the direct interaction between the Pto kinase and the CD loop (Tang *et al.*, 1996; Xing *et al.*, 2007). In tobacco, the CD loop is not involved but instead the CTD is recognized by a putative R protein (Shan *et al.*, 2000b). To further investigate the role of the CD loop and the CTD in allowing recognition of AvrPto we used *Agrobacterium*-infiltration to transiently express a series of AvrPto variants in leaves of tomato and *Nicotiana sylvestris* (a parental species of amphidiploid tobacco) (Figure 2.3A). Wild type AvrPto was recognized in both species, leading to visible cell death and the G2A substitution in AvrPto abolished this response. The I96A substitution, which disrupts the interaction between AvrPto and Pto (Tang *et al.*, 1996), abolished cell death in tomato RG-PtoR, as expected, but not in *N. sylvestris*.

AvrPto variants that either lack the CTD (D30) or have a CTD that is unable to be phosphorylated (S147A/S149A) did not elicit cell death in *N. sylvestris* (Figure 2.3A). Significantly, however, an AvrPto variant with aspartate substitutions at S147/S149, which mimic the negative charge of phosphorylated serine residues, does elicit cell death in *N. sylvestris*. AvrPto-mediated cell death in tomato RG-PtoR is unaffected in response to either the 2xA or ΔC30 alterations indicating that the phosphorylation of the CTD does not markedly affect Pto-mediated recognition of AvrPto. Collectively, these results indicate that phosphorylation of S147 and S149 is required for recognition of AvrPto by *N. sylvestris* but not by tomato RG-PtoR. The same set of AvrPto variants were also tested in *N. benthamiana* and the results were similar with those from RG-PtoR tomato suggesting that *N. benthamiana* possesses a Pto-like resistance specificity (Figure 2.4A). Protein expression in plant cells was demonstrated for all the AvrPto variants using *N. benthamiana* leaves (Figure 2.4B).

Cultivated tobacco is thought to have arisen from an ancient hybridization event between *N. sylvestris* and *N. tomentosa* (Chase *et al.*, 2003; Clarkson *et al.*, 2004). To further characterize the recognition of AvrPto in these species we expressed the AvrPto variants in *P. s.* pv. *tabaci* and inoculated leaves of these two species and tobacco using a high bacterial titer for an HR assay (4×10^7 cfu/mL; Figure 2.3B). An HR was elicited in all three species by AvrPto and the phosphomimic AvrPto(S147D/S149D). In *N. sylvestris* and *N. tabacum*, the HR was abolished when AvrPto contains either the 2xA or ΔC18, whereas AvrPto with the I96A substitution still elicited an HR. In contrast, *N. tomentosa* did not recognize the CD loop variant but did respond to the AvrPto CTD-minus variants. All AvrPto variants were expressed and secreted similarly from *P. s.* pv. *tabaci* (Figure 2.3C and D).

A disease assay using a lower titer (10^5 cfu/mL) of *P. s.* pv. *tabaci* was performed in all three *Nicotiana* species used for the HR assay (Figure 2.5A). We observed cell death associated with disease only in interactions involving the AvrPto proteins that did not cause an HR. Consistent with the observations from the HR assay, AvrPto containing either the 2xA or ΔC18 was not recognized by *N. sylvestris* or *N. tabacum* leading to disease symptom development, whereas the I96A substitution in AvrPto allowed disease formation in *N. tomentosa*.

To further assess the effects of CTD phosphorylation on recognition by tobacco we measured bacterial populations in *N. sylvestris* leaves inoculated with the *P. s.* pv. *tabaci* strains delivering AvrPto or the variants (Figure 2.5B). The phosphomimic protein, AvrPto(S147D/S149D), caused inhibition of bacterial growth to the same extent as wild type AvrPto and AvrPto(I96A). Interestingly, there was a quantitative effect associated with individual substitutions at the S147 and S149 residues. Other alterations affecting the CTD abolished recognition by *N. sylvestris*. Together, these observations indicate that the phosphorylation status of the CTD and not simply a

structural feature (Anderson *et al.*, 2006), is monitored by *N. sylvestris* and tobacco. We therefore now refer to the putative R protein in tobacco and *N. sylvestris* as Rpa: ***R****esistance to* ***p****hosphorylated* ***A****vrPto*.

Rpa is conserved in several tobacco species but is not present in tomato species

We next investigated how widespread Rpa recognition specificity is among wild relatives of tobacco (Table 2.1). We found that Rpa is present in multiple accessions of both *N. tabacum* and *N. sylvestris*. Two accessions tested of the other presumed progenitor of tobacco, *N. tomentosa*, did not recognize the CTD but rather, like tomato, recognized the CD loop. Therefore, tobacco appears to have retained the Rpa specificity from *N. sylvestris* but not the Pto-like specificity of *N. tomentosa*. Two other diploid tobacco species, *N. langsdorfii* and *N. rustica*, also express an Rpa specificity suggesting this recognition mechanism arose before *Nicotiana* speciation. Diverse wild relatives of tomato were also tested and none of them were found to recognize the CTD (Table 2.2).

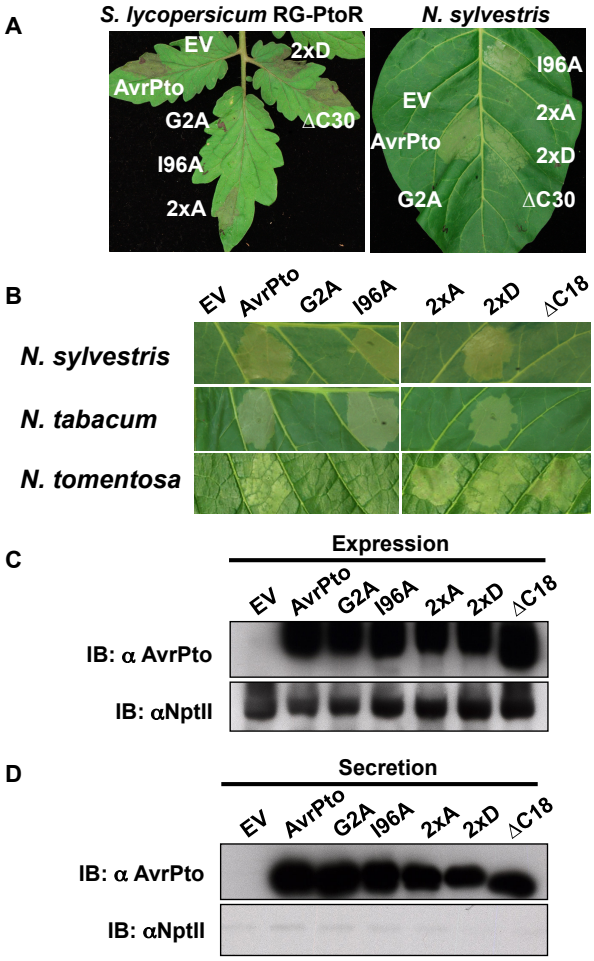

Figure 2.3. Rpa in tobacco recognizes the phosphorylated C-terminal domain of AvrPto. (A) AvrPto recognition assay in tomato and *N. sylvestris* using *Agrobacterium*-mediated transient expression. AvrPto, G2A, I96A, 2xA (S147A/S149A), 2xD (S147D/S149D) or ΔC30 were expressed in leaves of tomato RG-PtoR (*Pto/Pto Prf/Prf*) and *N. sylvestris*. EV indicates an empty vector control. Photographs were taken at 22 hours (tomato) or 20 hours (*N. sylvestris*) after inoculation. **(B)** HR assay in *N. sylvestris* 'TW136', *N. tabacum* 'W38' or *N. tomentosa* 'TW141' using *P. s.* pv. *tabaci* delivering the AvrPto proteins. A high bacterial titers ($4x10^7$ cfu/ml) were used for inoculation. Images were taken at 20 hours after inoculation. **(C)** Immunoblotting using an αAvrPto antibody was performed to detect expression of AvrPto proteins by *P. s.* pv. *tabaci* 11528R. **(D)** Immunoblotting using an αAvrPto antibody was performed to detect secretion of AvrPto from *P. s.* pv. *tabaci* 11528R.

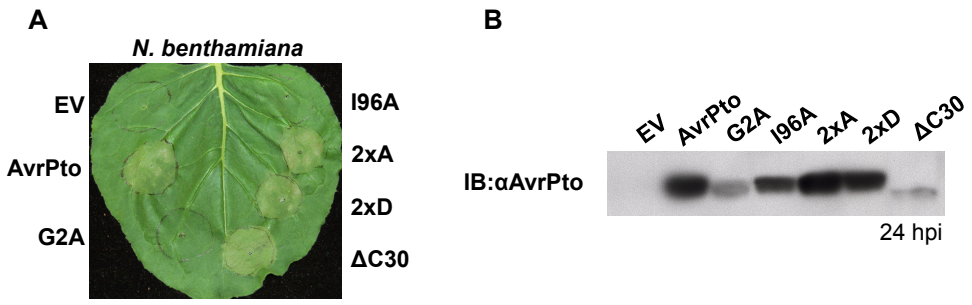

Figure 2.4: *Agrobacterium*-mediated transient expression assay in leaf of *N. benthamiana*. A) AvrPto, AvrPto(G2A), AvrPto(I96A), AvrPto(2xA; S147A/S149A), AvrPto (2xD; S147D/S149D) or AvrPto(ΔC30) were expressed in *N. benthamiana* leaf and a photograph was taken at 30 hours later. B) Expression of the AvrPto proteins was examined at 24 hours after infiltration by using an immunoblot assay with an αAvrPto antibody.

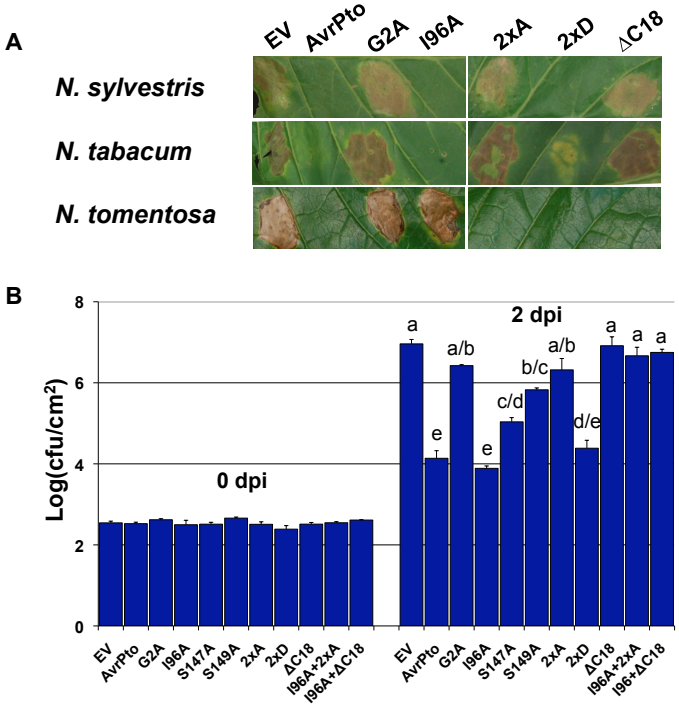

Figure 2.5. The recognition of the phosphorylated-Ser at position 147 and 149 by Rpa shows quantitative effects and is independent from the CD loop structure. **(A)** Disease assays in *N. sylvestris* 'TW136', *N. tabacum* 'W38' or *N. tomentosa* 'TW141' using *P. s.* pv. *tabaci* delivering the AvrPto proteins. Photographs of disease assays using low bacterial inoculum (10^5 cfu/mL) were taken at 7 days post-inoculation. Disease-associated cell death indicates lack of recognition. **(B)** Bacterial population assays in *N. sylvestris* 'TW136' at 2 dpi. *N. sylvestris* leaves were syringe-infiltrated with *P. s.* pv. *tabaci* delivering the AvrPto variants at 10^5 cfu/ml. Data are presented as colony-forming units (cfu) per square centimeter of leaf tissue. Letters represent groupings of statistical significance based on analysis of variance and comparisons for all pairs using Tukey-Kramer HSD ($P \leq 0.05$). Error bars indicate ± SE (n=3).

Table 2.1. Recognition of the C-terminal domain of AvrPto by wild species of tobacco using a disease assay. Leaves of each wild species were inoculated with *P. s.* pv. *tabaci* strains delivering empty vector, AvrPto, AvrPto(I96A) or AvrPto(2XA-S147A/S149A) (10^5 cfu/mL) and the host response was recorded 5 days after inoculation.

Species	Accessions	Empty Vector	AvrPto	AvrPto (I96A)	AvrPto (2xA)
N. tabacum	BY2	S	R	R	S
N. tabacum	KY14	S	R	R	S
N. tabacum	Petite Havana	S	R	R	S
N. tabacum	Samsun	S	R	R	S
N. tabacum	Virginia Bright	S	R	R	S
N. tabacum	Virginia Gold Leaf	S	R	R	S
N. tabacum	W38	S	R	R	S
N. tabacum	Xanthi	S	R	R	S
N. sylvestris	TW136	S	R	R	S
N. sylvestris	TW137	S	R	R	S
N. sylvestris	TW138	S	R	R	S
N. tomentosa	TW141	S	R	S	R
N. tomentosa	TW140	S	IS	S	IS
N. undulata		S	IS	IS	IS
N. langsdorfii		S	IS	IS	S
N. benthamiana[1]		S	IS	IS	IS
N. rustica		S	R	R	S

R = no disease symptoms were observed; IS = an intermediate degree of disease was observed; S = extensive disease symptoms were observed.

[1]Based on Agrobacterium-mediated cell death assay, *N. benthamiana* can recognize AvrPto and AvrPto (2xA), but not AvrPto (I96A).

Table 2.2. **Recognition of AvrPto by wild species of tomato using a disease assay.** Leaves of each species were inoculated with *P. s.* pv. *tomato* T1 strains delivering empty vector, AvrPto, AvrPto(I96A), or AvrPto(ΔC18) ($5*10^4$ cfu/mL). The host response to each strain was recorded 7 to 10 days after inoculation as either resistance (R) or susceptibility (S).

Accession[1]	Species	EV	AvrPto	AvrPto(I96A)	AvrPto(ΔC18)
LA0441	*S. arcanum*	S	S	S	S
LA0421	*S. cheesmaniae*	S	S	S	S
LA1028	*S. chmielewskii*	S	S	S	S
LA2663	*S. chmielewskii*	S	S	S	S
LA2677	*S. chmielewskii*	S	S	S	S
LA1136	*S. galapagense*	ND[2]	S	S	S
LA1141	*S. galapagense*	ND[2]	S	S	S
LA0361	*S. habrochaites*	S[4]	R[4]	S[4]	R[4]
LA1361	*S. habrochaites*	S[4]	R[4]	S[4]	R[4]
LA0407	*S. habrochaites*	S	R	S	R
LA0247	*S. neorickii*	S	S	S	S
LA1322	*S. neorickii*	S	S	S	S
LA1329	*S. neorickii*	IS[3]	S	S	S
LA1626	*S. neorickii*	S[4]	R[4]	S[4]	R[4]
LA1716	*S. neorickii*	IS[3]	S	S	S
LA0716	*S. pennellii*	S	S	S	S
LA2657	*S. pennelli*	S	S	S	S
LA0750	*S. pennellii*	S	S	S	S
LA1920	*S. pennellii*	S	S	S	S
LA0114	*S. pimpinellifolium*	S	S	S	S
LA0373	*S. pimpinellifolium*	S[4]	R[4]	S[4]	R[4]
LA0411	*S. pimpinellifolium*	S	S	S	S
LA1617	*S. pimpinellifolium*	S	S	S	S

[1]Wild species were obtained from the C. M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/).
[2]ND= not determined.
[3]IS = an intermediate degree of disease was observed.
[4]In these cases, recognition of AvrPto was also confirmed by Agrobacterium-mediated cell death assays using the indicated AvrPto constructs. Expression of AvrPto or AvrPto (ΔC18) caused cell-death, while expression of AvrPto (I96A) did not.

Discussion

AvrPto is a well-characterized effector and yet it continues to reveal new information about how it manipulates the plant cell and, in turn, how the plant has evolved to counter these virulence activities (Navarro *et al.*, 2008; Shan *et al.*, 2008; Xiang *et al.*, 2008). We have shown here that AvrPto has a modular structure and that its phosphorylation by a host kinase contributes in an additive fashion to its virulence activity and also to its recognition by a tobacco R protein. This modular structure consists first of an N-terminal region which is required for secretion of AvrPto into the plant cell and for its localization to the plant plasma membrane (Chang *et al*., 2000; Shan *et al*., 2000; Chang *et al*., 2001). Beyond this, AvrPto contains two other structurally discrete domains, the CD loop (Wulf *et al*., 2004; Xing *et al*., 2007) and the CTD, each of which has a distinct virulence activity. The CTD appears to exploit a host protein kinase in order to facilitate its virulence activity but certain *Nicotiana* species have evolved to specifically detect this manipulation. Although the CTD enhances bacterial growth in tomato, to date, no tomato species are known that are able to recognize this domain to activate immunity. The fact that certain *Nicotiana* species do recognize the phosphorylated CTD suggests this domain plays an important role in bacterial pathogenesis of *Nicotiana*.

Our initial observation that the CD loop and the CTD each contribute in an additive fashion to bacterial growth, disease symptoms, and ethylene production suggested that these two domains may act via different mechanisms. This possibility was tested by using an assay in which AvrPto suppresses flg22-mediated activation of a host MAPK. This suppression is known to involve the CD loop which is required for interaction with the kinase domains of both FLS2 and BAK1 thereby disrupting downstream signaling (Shan *et al.*, 2008; Xiang *et al.*, 2008). A previous report examined two alterations in the CTD (P146L and S147R) and found that they did not

affect MAPK suppression activity (He *et al.*, 2006) although neither of these alterations disrupted both S147 and S149. We found here that such a dual mutant (S147A/S149A) is able to fully suppress flg22-mediated MAPK activation and this now excludes a role for CTD phosphorylation in suppression of this PTI response. Furthermore, S147/S149 phosphorylation was not required for suppression of MAPK activation in response to another PAMP, chitin. These results indicate that the CTD promotes virulence in a manner distinct from the CD loop, which appears to interfere with multiple PTI pathways. The mechanism by which the CTD promotes bacterial virulence is unknown and it remains an open question whether it also suppresses certain PTI responses or, alternatively, may act as a positive regulator to enhance a host process resulting in susceptibility.

Of the relatively few *P. syringae* type III effectors that have been studied in detail, three have now been shown to be phosphorylated by host kinases (AvrB, AvrPto, and AvrPtoB) (Zipfel *et al.*, 2004; Desvaux *et al.*, 2007; Xiao *et al.*, 2007a). Phosphorylation, along with myristylation, therefore appears to be a common post-translational modification of effectors upon their delivery into the plant cell. Several possibilities exist for the molecular basis of CTD phosphorylation. For example, many host kinase genes are transcriptionally induced upon exposure of plant cells to bacterial PAMPs and AvrPto might take advantage of a PTI-induced kinase to phosphorylate its CTD (Navarro *et al.*, 2004; Cohn and Martin, 2005). That kinase could, in turn, be a virulence target of AvrPto. It is also possible that the interaction between AvrPto and the CTD kinase leads to activation of downstream targets that promote AvrPto virulence. Ultimately, identification and characterization of the host kinase will be needed to shed light on the molecular basis of its interaction with AvrPto, its contribution to *Pseudomonas syringae* virulence, and its normal role in plants when it is not phosphorylating AvrPto.

Plant recognition of a specific effector protein will obviously be more durable and effective if the host recognition mechanism targets the domain of the effector that is required for its virulence activity. There are now many cases where both the avirulence and virulence activities of an effector are coupled in this way; the contribution of the CD loop to both virulence and recognition by Pto exemplifies this relationship (Scofield *et al.*, 1996; Tang *et al.*, 1996; Kim *et al.*, 2002; Xing *et al.*, 2007). Remarkably, AvrPto now provides another example of this coupling in which the phosphorylated CTD plays both a role in virulence and is targeted by a recognition mechanism in tobacco. We are currently unable to test the virulence activity of the CTD in tobacco because we have not identified an accession that lacks Rpa-mediated resistance (and technical difficulties have prevented us from using *N. tomentosa* TW141 to address this point). However, based on the fact that the phosphorylated CTD promotes virulence in tomato we hypothesize that it will also do so in tobacco. Indeed, it is reasonable to assume that such virulence activity provided the selection pressure giving rise to Rpa in *Nicotiana* species.

N. tabacum is a complex amphidiploid that is thought to have originated from a natural hybridization event between the two diploid species, *N. sylvestris* and *N. tomentosa* (Chase *et al.*, 2003). Since *N. sylvestris* expresses a specificity for the CTD (Rpa) and *N. tomentosa* recognizes the CD loop (i.e., it has a Pto-like activity), *N. tabacum* might be expected to have both Rpa and Pto-like specificities. However, we observed only Rpa specificity in the eight *N. tabacum* accessions we examined. It is possible that the specific *N. tomentosa* plant involved in the original hybridization event leading to tobacco did not have *Pto*. Alternatively, it is possible that *Pto* was lost from *N. tabacum* either randomly, or because *Rpa* alone has been sufficient to provide durable resistance against bacterial pathogens delivering AvrPto (note that only one of the specificities, *Pto*, is observed among the accessions of tomato wild

species). In the future, identification of the *Rpa* gene from *N. sylvestris* and the putative *Pto* gene from *N. tomentosa* should allow a deeper understanding of the evolutionary history and mechanistic differences of these two recognition specificities.

AvrPto has both remarkable similarities and striking differences with AvrPtoB, the other type III effector recognized by the tomato Pto kinase. Both effectors make physical contact with the Pto P-loop (Xing *et al.*, 2007; Dong *et al.*, 2009) although they each have another unique contact surface involved in binding to Pto. AvrPto is only 18 kD while AvrPtoB is 60 kD and their structures are markedly different (Kim *et al.*, 2002; Wulf *et al.*, 2004; Janjusevic *et al.*, 2006; Xing *et al.*, 2007; Dong *et al.*, 2009). However, the virulence activity of both effectors is enhanced by a host-mediated phosphorylation event although it is not known whether this modification is unnecessary for AvrPtoB disruption of the FLS2/BAK1 complex as it is for AvrPto (Anderson et al., 2006; Xiao et al., 2007b). Each effector is known to be recognized by two R proteins – Pto and Fen in the case of AvrPtoB (Kim *et al.*, 2002; Rosebrock *et al.*, 2007) and Pto and Rpa in the case of AvrPto (Martin *et al.*, 1993; Shan *et al.*, 2000b). It is also striking that AvrPto, like AvrPtoB, has a modular structure with discrete domains having distinct virulence activities. The extent to which type III effectors as a whole display modular structures is unknown but the fact that some of these proteins are large and have diverse activities might indicate that modularity is a common feature of this important class of pathogen virulence factors.

Experimental procedures

Protoplast assays for MAP kinase suppression

Three- to four-week-old tomato Rio Grande-prf3 (*Pto/Pto prf/prf*) or Rio Grande-PtoS (*pto/pto Prf/Prf*) leaves were used for protoplast isolation. Protoplasts were

transformed using a polyethylene glycol protocol described previously ((Rosebrock et al., 2007; Xiao et al., 2007c); http://genetics.mgh.harvard.edu/sheenweb/). The HA-tagged *SlMPK3* gene (Holley *et al.*, 2003) was expressed in protoplasts by using the pTEX CaMV 35S promoter expression cassette (Xiao et al., 2007c). AvrPto variants were expressed with a CaMV 35S promoter in vector pJD301 (Anderson *et al.*, 2006). The *avrPto* gene from *P. s.* pv. *tomato* strain JL1065 was used as the template for all experiments in this paper. Ten ug of pTEX::*SlMPK3* and 7 ug of pJD301::*avrPto* plasmids were used in each transformation. After 6 hours of incubation, PAMP-treatments were as follows: 100 nM flg22 (GenScrip) or 50 µg/mL chitin (Sigma-Aldrich Inc.). Ten minutes after PAMP treatment, the protoplasts were collected by centrifugation. Detection of the AvrPto-mediated suppression of PAMP-induced MAPK activity was performed as described previously (He *et al.*, 2006). Data shown are representative of a minimum of three independent experiments.

Pathogenesis assays in tomato and tobacco

For the pathogenesis assays in tomato, the *avrPto* variants were cloned into the broad-host-range vector pCPP45 (Lin and Martin, 2005) and transformed into *P. s.* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* by electroporation. For the pathogenesis assays in tobacco, the *avrPto* variants were cloned into the broad-host-range vector pDSK519 (Anderson *et al.*, 2006), and transformed into *P. s.* pv. *tobacco* 11528R. Expression and secretion assays of the AvrPto variants for both *P. s.* pv. *tomato* and *P. s.* pv. *tabaci* were performed using published protocols (Shan *et al.*, 2000b). Site-directed mutagenesis of AvrPto was performed using the Quickchange protocol and Pfu Turbo DNA polymerase (Stratagene). Primers are listed in Table 2.3. The antibodies used for immunoblotting were anti-AvrPto (Shan *et al.*, 2000a) and anti-NptII (U.S. Biological Corp.).

Five- to six-week-old plants of tomato Rio Grande-prf3 (*Pto*/*Pto, prf*/*prf*) were vacuum-infiltrated with different *P. s*. pv. *tomato* DC3000 strains at an inoculum of 10^4 colony-forming units/mL and maintained in a climate-controlled growth chamber as described previously (Anderson *et al.*, 2006). Bacterial populations in tomato leaves were measured at 2 or 3 days after infiltration. Analysis of variance (ANOVA) and comparisons for all pairs using Tukey-Kramer HSD were performed using JMP7 (SAS Institute Inc.). The least significance difference at a 0.05 probability level was used to test the differences between means. Error bars indicate standard error (n=4). Disease symptoms were photographed 5 days after inoculation.

Four- to five-week-old tobacco plants (*N. sylvestris*, *N. tomentosa*, or *N. tabacum*) were used for inoculating different *P. s*. pv. *tabaci* 11528R strains by syringe infiltration. For disease assays, an inoculum of 10^5 cfu/mL was used, and for HR assays an inoculum of $4x10^7$ cfu/mL was used. Bacterial populations were measured in the disease assay 2 or 3 days after inoculation. JMP7 was used for statistical analysis with the least significance difference at a 0.05 probability level. Error bars indicate standard error (n=3). Disease symptoms were photographed 5 days after inoculation. Plant responses were photographed at 7 days after inoculation for the disease assays and at 20 hours after inoculation for the hypersensitive response (HR). Data shown represent a minimum of three independent experiments.

Agrobacterium-mediated transient expression

A. tumefaciens strain GV2260 was used to deliver the pCAMPBIA2300 with a CaMV 35S promoter expression cassette for transient gene expression. All AvrPto variants contain a C-terminal HA epitope tag. Presence or absence of cell-death caused by overexpressing AvrPto protein was determined at 20 hours post inoculation (hpi) for *N. sylvestris* and *N. tabacum*, 22 hpi for *S. lycopersicum*, and 30 hpi for *N.*

Table 2.3. Primers used in this study.

Primer name	Sequence (5' → 3')
AvrPtoF	CACCATGGGAAATATATGTGTCGG
AvrPtoR	TTGCCAGTTACGGTACGGGCTA
AvrPto**2XA**F*	CGACTATGAACCCGGCCGGAGCTATTCGAATGTCAAC
AvrPto**2XA**R*	GTTGACATTCGAATAGCTCCGGCCGGGTTCATAGTCG
AvrPto**3XA**F*	ATGAACCCGGCCGGAGCTATTCGA ATGGCAACAC
AvrPto**3XA**R*	GTGTTGCCATTCGAATAGCTCCGGCCGGGTTCAT
AvrPto**2XD**F*	GCGACTATGAACCCGGATGGAGATATTCGAATGTCAAC
AvrPto**2XD**R*	GTTGACATTCGAATATCTCCATCCGGGTTCATAGTCGC

*Amino acid substitutions were made using PCR-based (Pfu turbo tag polymerase) side-directed mutagenesis reactions with these primer pairs (amino acid changes in bold).

benthamiana. Data shown represent a minimum of three independent experiments. Expression of AvrPto proteins was confirmed in *N. benthamiana* 24 hpi, prior to visible cell-death appearance. The antibodies used for immunoblotting were anti-AvrPto (Shan *et al.*, 2000a) or anti-HA (Roche Applied Science).

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CHAPTER 3

TWO VIRULENCE DETERMINANTS OF TYPE III EFFECTOR AVRPTO ARE FUNCTIONALLY CONSERVED IN DIVERSE *PSEUDOMONAS SYRINGAE* PATHOVARS[3]

Abstract

The *Pseudomonas syringae* pv. *tomato* type III effector protein AvrPto has two functional domains that contribute additively to its ability to promote pathogen virulence in susceptible tomato plants and also elicit defense responses in resistant tomato and tobacco genotypes. Here we test the hypothesis that key amino acid residues in these two domains will be conserved even in sequence-divergent AvrPto proteins expressed by diverse *P. syringae* pathovars. We cloned *avrPto* homologs from diverse *P. syringae* pathovars and characterized the four most diverse homologs from *P. syringae* pathovars *mori, lachrymans, myricae,* and *oryzae* for their virulence activity and ability to elicit resistance in tomato and tobacco. Key residues within the two AvrPto domains are conserved in three of the four homologs and are required for virulence activity and defense elicitation. AvrPto$_{oryzae}$, lacks conserved residues in each domain, but was found to be recognized by a previously unknown resistance gene in both tomato and tobacco. Our results indicate that the two virulence domains of AvrPto are conserved in diverse pathovars despite the fact these domains are recognized by certain plant species. AvrPto may therefore function in pathovars infecting diverse plant species by targeting conserved host processes.

[3] Adapted from H. P. Nguyen, I. Yeam, A. Angot, and G. B. Martin. 2010. Two virulence determinants of type III effector AvrPto are functionally conserved in diverse *Pseudomonas syringae* pathovars. New Phytologist (*in press*).

Introduction

Among bacterial pathogens of plants, *Pseudomonas syringae* has been extensively studied due to its ability to infect a wide range of economically important crops. *P. syringae* is classified into ~50 pathovars based on their ability to cause disease on different host plants. One of the most important strategies that successful pathogenic bacteria employ to subvert host defense pathways and to promote bacterial growth is the injection of a suite of virulence proteins, termed effectors, into the host cell cytoplasm via the type III secretion system (T3SS), a specialized injection apparatus used by many γ-proteobacteria (Alfano and Collmer, 2004; Cunnac et al., 2009). Type III effectors (T3Es) play central roles in promoting bacterial virulence on susceptible host plants (Chisholm *et al.*, 2006). In resistant plants, however, some of these effectors act as elicitors of a strong immune response, usually associated with localized programmed cell death (the hypersensitive response; HR), through their direct or indirect recognition by resistance (R) proteins (Chisholm *et al.*, 2006). The outcome of the interaction between the host plant and its microbial pathogen therefore depends on the ability of the T3Es to overcome host defenses and whether or not the host recognizes the presence of certain effectors leading to a strong defense response. This complex multi-layered interplay suggests that T3Es and the host defense system are involved in a co-evolutionary 'arms race' where both sides impose strong selective pressure on one another.

To date, the genomes of six *P. syringae* strains belonging to five different pathovars have been fully sequenced or draft-assembled and annotated, including *P. s. tomato* (*Pst* strain DC3000), *P. s. syringae* B728a (*Psy*), and *P. s. phaseolicola* 1448A (*Pph*), *Pst* T1, *P.s. oryzae* 1_6 (*Por*), and *P. s. tabaci* 11528 (*Pta*) (Buell *et al.*, 2003; Lindeberg *et al.*, 2008; Almeida *et al.*, 2009; Reinhardt *et al.*, 2009; Studholme *et al.*, 2009). These pathovars encompass representatives from four major monophyletic

groups within the species *P. syringae* with *Pph* and *Pta* being from the same group (Studholme *et al.*, 2009). Analysis of these genomes revealed that each strain has about 30 effectors, with some shared among most strains, representing a common effector core while others are variable, possibly contributing to the specific bacterium-host interactions (Lindeberg *et al.*, 2008). Due to functional redundancy or possibly because they have no function in the plant host tested, deletion of most individual effectors has little or no effect on bacterial virulence (Kvitko *et al.*, 2009).

Only a few effectors have been experimentally shown to have a major contribution to the bacterial virulence in host plants, including AvrPto (AvrPto1), AvrPtoB (HopAB2), AvrE1, and HopM1 (Lin and Martin, 2005; Badel et al., 2006). Of these, AvrPto is the longest, most extensively studied and best characterized effector. Recent studies have shown that AvrPto promotes disease in susceptible hosts by interfering with receptor complexes and blocking signal transmission required for the first line of host defense, pathogen-associated molecular pattern (PAMP)-triggered immunity, or PTI (He *et al.*, 2006; Shan *et al.*, 2008). In plants expressing the resistance protein complex, Pto/Prf, AvrPto is recognized via a direct interaction with the Pto kinase activating effector-triggered immunity (ETI) (Tang *et al.*, 1996; Xing *et al.*, 2007).

Once injected into the host cell via the T3SS, AvrPto, a small (18 kDa) hydrophilic protein (Ronald *et al.*, 1992), undergoes host-mediated post-translational modifications including phosphorylation and N-terminal myristoylation to be targeted to the plant plasma membrane where it promotes virulence (Shan *et al.*, 2000b; Anderson *et al.*, 2006). AvrPto has been subjected to extensive site-directed mutagenesis studies, which have demonstrated that individual substitutions of ~80% of its amino acid residues show no detectable effect on its virulence activity or recognition by the host (Shan *et al.*, 2000a; Chang *et al.*, 2001; Anderson *et al.*, 2006;

Pascuzzi, 2006). The results of these studies revealed that two structurally distinct functional domains, in addition to the N-terminal myristoylation motif and type III secretion signal, contribute to AvrPto virulence and its recognition by the host.

The first AvrPto virulence domain is termed the CD loop, for the omega (Ω) loop located between α-helices C and D in the core structure (Wulf *et al.*, 2004). Residues in this loop mediate the interaction of AvrPto with the kinase domains of its virulence targets, pattern recognition receptor (PRR) complexes such as FLS2/BAK1, which inhibits their activity (He *et al.*, 2006; Xiang *et al.*, 2008). The CD loop also participates in a direct physical interaction with the Pto kinase, which inhibits Pto kinase activity and activates the Prf-dependent ETI defense response (Xing et al., 2007). Since substitutions made in certain residues in the CD loop abolishing AvrPto recognition by Pto also reduce AvrPto virulence, it has been suggested that Pto might have evolved as a 'molecular mimic' of the CD loop virulence targets (Xing *et al.*, 2007; Xiang *et al.*, 2008).

The second virulence domain of AvrPto is the phosphorylated C-terminal domain (CTD). The phosphorylated residues within the CTD, in particular, serine residues S147 and S149, are responsible for its virulence activity (Anderson *et al.*, 2006; Yeam *et al.*, 2009). The CTD promotes virulence via an unknown but distinct mechanism from that of the CD loop *(Yeam et al.*, 2009). The CD loop and CTD were recently shown to contribute in an additive fashion to AvrPto virulence; mutations in either one reduce virulence activity while alterations of both together abolish AvrPto virulence (Yeam *et al.*, 2009). As an interesting corollary with CD loop recognition by Pto, the phosphorylation status of the CTD is monitored by a second distinct recognition mechanism termed Rpa (Recognition of phosphorylated AvrPto) in various tobacco species (Yeam *et al.*, 2009) .

AvrPto-related sequences are found in many pathovars of *P. syringae* that infect a wide range of host plants (Sarkar et al., 2006; Lin and Martin, 2007). Strains that express AvrPto (or AvrPtoB) can grow and cause pathovar-specific disease symptoms on susceptible tomato plants whereas they elicit resistance on tomato expressing Pto and Prf (Lin and Martin, 2007). This suggests that these AvrPto homologs are functional and might contribute to the fitness of other *P. syringae* pathovars. However, since both AvrPto and AvrPtoB are present in many of these pathovars, the extent to which AvrPto itself contributes to bacterial virulence functions is unclear. Nevertheless, the fact that plants have evolved two distinct recognition mechanisms to monitor the two virulence determinants of AvrPto suggests that the CD loop and the CTD might be important and functionally conserved in AvrPto proteins that are present in other *P. syringae* strains (Yeam *et al.*, 2009). Previous characterization of homologs of the effector AvrPtoB, showed that despite having divergent sequences, these homologs displayed conserved virulence functions on susceptible tomato plants while being recognized by Pto/Prf in resistant plants (Lin *et al.*, 2006). This supports an important role for positive selection on key virulence determinants of AvrPtoB. Previous studies looking at the diversity of homologs of T3Es found in other *P. syringae* pathovars showed that most effectors have undergone purifying selection probably to maintain certain virulence functions, adapt to specific host targets, or to avoid host recognition (Rohmer *et al.*, 2004).

We hypothesized that due to their important roles in AvrPto function, the CD loop and CTD virulence domains will be functionally conserved in diverse *P. syringae* pathovars. Here we examined this possibility by cloning and characterizing highly diverse AvrPto homologs from various *P. syringae* strains including the pathovars infecting cucumber (pv. *lachrymans*), rice (pv. *oryzae*), mulberry (pv. *mori*), and

bayberry (pv. *myricae*). Our results provide evidence that the CD loop and the CTD are indeed functionally conserved and contribute to the virulence activity of AvrPto homologs while also eliciting recognition in bacterial speck resistant accessions of tomato and tobacco.

Results

AvrPto homologs are present and expressed in diverse *Pseudomonas syringae* pathovars

To determine the presence of *avrPto*-related sequences in various *P. syringae* pathovars, we examined 78 strains belonging to 13 different *P. syringae* pathovars available in our and Dr. D. Guttman's (U. of Toronto) laboratories (Sarkar and Guttman, 2004) as well as *Pst* strains collected recently from fields in New York and Florida. First, we used $avrPto_{tomato\text{-JL1065}}$ to probe a DNA gel blot to detect the presence of *avrPto*-related sequences. Of the *P. syringae* pathovars tested, 33 strains (42%) belonging to 8 pathovars had an *avrPto*-related sequence (Figure 3.1 and Table 3.2). Some of the pathovars that were reported to have *avrPto*-related sequences in a previous DNA microarray study (Sarkar *et al.*, 2006) were not confirmed, possibly due to the higher hybridization stringency we used here.

In order to obtain their DNA sequences and to examine activities of the AvrPto homologs, we cloned 30 *avrPto* genes from selected strains of *P. syringae* (Table 3.1). *AvrPto* homologs from multiple isolates within pathovars *tomato, maculicola*, and *syringae* were successfully PCR-amplified using $avrPto_{tomato}$-specific primers (Table 3.1). Homologs from pathovars *lachrymans, papulans, mori, myricae,* and *oryzae* were unable to be retrieved using the $avrPto_{tomato}$ primer set and were instead cloned

from bacterial genomic libraries (Materials and Methods). Sequences of AvrPto homologs from isolates within individual pathovars were found to be identical with the exception of AvrPto$_{tomato\text{-DC3000,}}$ which has four single nucleotide polymorphisms, leading to four amino acid changes, as compared to other AvrPto$_{tomato}$ sequences (Figure 3.2A).

We investigated by RT-PCR using primers specific for each homolog (Table 3.3) and by protein gel blot using an antibody specific for AvrPto$_{tomato}$ whether *P. syringae* strains carrying *avrPto*-related sequences actually express the gene and corresponding protein. RT-PCR confirmed the RNA expression of *avrPto* in each pathovar with the exception of *avrPto*$_{oryzae}$ (Table 3.1). Protein gel bots detected AvrPto proteins in all pathovars except *myricae* and *oryzae* (Table 3.1). The weaker detection or apparent absence of AvrPto in some strains may be due to a lack of antigen homology of these AvrPto homologs to the AvrPto$_{tomato}$ antibody used in this assay. As for AvrPto$_{oryzae}$, despite the presence of an open reading frame, these experiments suggested this allele may not be expressed or is expressed at a very low level in *P. s.* pv. *oryzae*.

Key amino acid residues in the CD loop and the CTD are conserved in AvrPto homologs

Since AvrPto homologs from isolates within individual pathovars are identical in both nucleotide and amino acid sequences (with the exception of AvrPto$_{tomato\ DC3000}$) we used one representative isolate from each pathovar for subsequent sequence alignment and phylogenetic analysis (Figure 3.2A and B). AvrPto$_{syringae}$ and AvrPto$_{maculicola}$ have above 90% amino acid identity with AvrPto$_{tomato\ JL1065}$, whereas AvrPto homologs from other pathovars share much less sequence identity; AvrPto$_{mori}$ (70% identity), AvrPto$_{lachrymans}$ (73%), AvrPto$_{papulans}$(73%), AvrPto$_{myricae}$ (36%), and AvrPto$_{oryzae}$ (47%). AvrPto$_{papulans}$ is identical to AvrPto$_{lachrymans}$. A previous study reported that

avrPto-related sequences were not detected in *P. syringae* pv. *tabaci* by using a Southern blot assay (Lin and Martin, 2007). However, a recently published draft genome sequence of *P. syringae* pv. *tabaci* strain 11528 revealed an *avrPto* homolog whose protein would have 41% sequence identity with AvrPto$_{tomato}$ and this sequence was therefore also included in our analyses (Studholme *et al.*, 2009).

The N-terminal region containing the myristoylation motif and the type III secretion signal (Schechter *et al.*, 2004) is more conserved (98% identical) in the AvrPto homologs than the rest of the protein sequence (85.4% identical; Figure 3.2A and Supplemental Methods). The CD loop region of the AvrPto homologs (excluding AvrPto$_{oryzae}$ and AvrPto$_{tabaci}$) is also slightly more conserved (88%) than the rest of the protein sequence (84.7%, excluding the N-terminal region; Figure 3.2A and Supplemental Methods). Based on previous mutagenesis studies, however, many of the polymorphisms observed throughout the protein sequences of the AvrPto homologs as compared to AvrPto$_{tomato}$ would not be expected to cause changes in AvrPto$_{tomato}$ virulence activity or recognition by Pto (Shan *et al.*, 2000a; Chang *et al.*, 2001; Pascuzzi, 2006). A valine at position 96 (instead of an isoleucine at this position as in AvrPto$_{tomato}$), occurs in AvrPto$_{mori}$ and AvrPto$_{lacrymans}$. However, a I96V substitution in AvrPto has been shown previously to not disrupt the CD loop structure that is required for its virulence function and for its Pto-mediated recognition (Pascuzzi, 2006). Indeed, these two homologs interacted with the Pto kinase in a yeast two-hybrid assay (Table 3.2). On the other hand, AvrPto$_{myricae}$ and AvrPto$_{oryzae}$, which contain multiple polymorphisms in the CD loop as compared with AvrPto$_{tomato}$ did not interact with the Pto kinase in yeast.

In the CTD, the two critical serines, S147 and S149, are present in the majority of AvrPto homologs. S149 is present in all of them except AvrPto$_{oryzae}$ whereas S147 is present in all of them except for AvrPto$_{oryzae}$ and AvrPto$_{tomato\ DC3000}$ (Figure 3.2A). A

recent study indicated that AvrPto has to be in a partially unfolded form in order to be translocated into the host cell cytoplasm via the T3SS (Dawson *et al.*, 2009). This work also revealed that AvrPto possesses an intrinsic pH-sensitive switch controlled by the residue H87 that allows it to fold and unfold precisely in the pH range corresponding to the bacterial and host cell cytoplasm environments. All AvrPto homologs in this study possess the pH folding switch H87 indicating these proteins likely use the same mechanism to be delivered into the host cell cytoplasm.

AvrPto$_{tabaci}$ has many polymorphisms throughout its protein sequence including the CD loop and the CTD although it has a conserved S149. As expected, this homolog has been reported to not trigger Pto/Prf-dependent disease resistance (Studholme *et al.*, 2009). Phylogenetic analysis of these AvrPto homologs indicates the relative closeness between these homologs (Figure 3.2B). Notably, AvrPto$_{tabaci}$ appears to be the most distantly related from the other AvrPto homologs. For the functional analysis described below, we focused on homologs from pathovars *mori, lachrymans, myricae,* and *oryzae*.

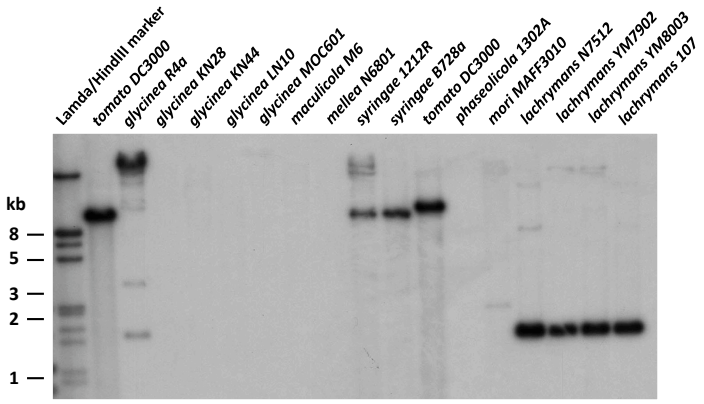

Figure 3.1. AvrPto homologs occur in diverse *P. syringae* pathovars. A representative DNA gel blot using *Pst* strain JL1065 *avrPto* DNA as a probe to detect the presence of *avrPto*-like sequences in different *P. syringae* pathovars. Blot shown contains bacterial genomic DNA digested with *HindIII*.

Table 3.1. Characteristics of *P. syringae* pathovars that have an *avrPto* homolog identified in this study. Strains examined further in this paper are shown in bold.

***P. syringae* pathovar**	**Strain designation**	**Host**	**DNA[1]**	**RNA[2]**	**Protein[3]**
coronofaciens	KN221	Oat	+	ND	ND
glycinea	R4a	Soybean	+	ND	ND
lachrymans	**N7512**	**Cucumber**	**+***	**+**	**±[4]**
lachrymans	YM7902	Cucumber	+	ND	±[4]
lachrymans	YM8003	Cucumber	+*	+	±[4]
lachrymans	107	Cucumber	+*	+	±[4]
maculicola	4981	Cauliflower	+*	+	+
maculicola	KN203	Chinese cabbage	+*	+	+
mori	**MAFF301020**	**Mulberry**	**+***	**+**	**±[4]**
mori	PDDCC4331	Mulberry	+*	+	±[4]
myricae	**MAFF302941**	**Bayberry**	**+***	**+**	**-**
myricae	AZ84488	Bayberry	+*	ND	-
oryzae	**36_1**	**Rice**	**+***	**-**	**-**
oryzae	I_6	Rice	+*	ND	-
papulans	5	Apple	+*	ND	ND
syringae	1212R	Pea	+*	+	+
syringae	B728A	Snap bean	+*	+	+
syringae	Ps9220	Spring onion	+*	ND	ND
tomato	DC3000	Tomato	+*	+	+
tomato	DCT6D1	Tomato	+*	+	+
tomato	DC84_1	Tomato	+*	+	+
tomato	DC89_4H	Tomato	+*	+	+
tomato	Bakerfield	Tomato	+*	+	+
tomato	PT11	Tomato	+*	+	+
tomato	PT23	Tomato	+*	+	+
tomato	133	Tomato	+*	+	+
tomato	1108	Tomato	+*	+	+
tomato	NYS race 0	Tomato	+*	+	+
tomato	409	Tomato	+*	+	+
tomato	A1	Tomato	+*	+	+
tomato	Gilreath #4	Tomato	+*	+	+
tomato	Gilreath #6	Tomato	+*	+	+
tomato	J.Scott	Tomato	+*	+	+

Table 3.1 (Continued):

ND: not determined
[1] The presence of *avrPto*-related sequence in each strain was determined by Southern blot using *avrPto*$_{tomato\ JL1065}$ DNA as a probe.
[*] These *avrPto* alleles were cloned and sequence verified in this study.
[2]RT-PCR was used with primer sets designed based on the *avrPto* sequence from the indicated pathovar. Symbol + indicates transcript was detected. The expression of 23S rRNA was used as an internal control.
[3]Protein from each pathovar was analyzed on an immunoblot using an AvrPto$_{tomato}$ antibody. Symbols indicate presence (+), weak detection (±) or absence (-) of the AvrPto protein.
[4]Cases where AvrPto was detected weakly or not at all may be due to a lack of epitopes to anti-AvrPto$_{tomato}$ in that pathovar's AvrPto protein.

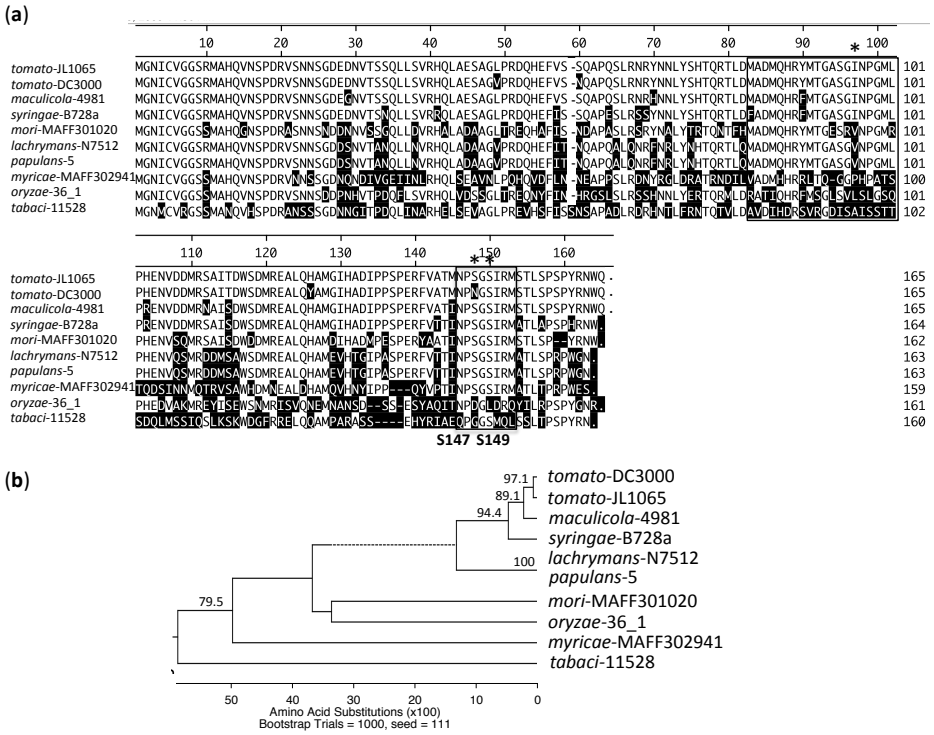

Figure 3.2. Sequence alignment and phylogenetic analysis of AvrPto homologs. **(A)** Amino acid sequence alignment of AvrPto homologs that were cloned in this study or publicly available. The CD loop region (residues 83 to 102) is shown in a box and residue I96 is marked with an asterisk. The two phosphorylated serines, S147 and S149 are marked with asterisks and the CTD (residues 145 to 152) is shown in a box. Residues shown in black boxes are different from those in AvrPto$_{tomato\text{-}JL1065}$. **(B)** Phylogenetic tree showing the evolutionary relationships among AvrPto homologs predicted from the multiple sequence alignment. The length of each pair of branches represents the distance between each sequence pair while units at the bottom of the trees indicate the number of substitution events. The dotted line indicates a negative branch length. The bootstrap support is indicated above each node. Bootstrap values under 60% are not presented.

AvrPto homologs elicit Pto-dependent cell death when transiently expressed in *Nicotiana benthamiana*

In *Nicotiana benthamiana*, co-expression of AvrPto$_{tomato}$ with the Pto kinase triggers programmed cell death (PCD) (Scofield *et al.*, 1996; Tang *et al.*, 1996). The PCD is known to be mediated by the direct interaction between the Pto kinase and the CD loop of AvrPto (Xing *et al.*, 2007). In order to determine which AvrPto homologs may be capable of Pto-mediated recognition, we transiently co-expressed the AvrPto homologs and Pto in *N. benthamiana* leaves using *Agrobacterium*-infiltration. AvrPto$_{tomato}$, AvrPto$_{mori}$ and AvrPto$_{lachrymans}$ (Table 3.1) each caused PCD in *N. benthamiana* leaves when co-expressed with Pto (Figure 3.3A). The CTD does not contribute to Pto recognition in this assay and, as expected, an AvrPto$_{tomato}$ variant with a disrupted CTD (AvrPto$_{tomato\ 2xA}$) caused PCD when co-expressed with Pto. No cell death was observed for the empty vector (EV) control, AvrPto with the disrupted CD loop AvrPto$_{tomato\ I96A}$, AvrPto$_{oryzae}$ or AvrPto$_{myricae}$ (Figure 3.3A). Notably, all AvrPto homologs that interact with Pto in the yeast two-hybrid assay (Table 3.2) caused PCD when co-expressed with Pto in *N. benthamiana*. These results indicate that the interactions between the AvrPto homologs and Pto in yeast two-hybrid assay directly correlate with their recognition by Pto in *planta.* Together, these observations suggest that AvrPto$_{mori}$ and AvrPto$_{lachrymans}$ have a CD loop structure that allows for the physical interaction between AvrPto and Pto, while AvrPto$_{myricae}$ and AvrPto$_{oryzae}$ do not. Weak cell death was also observed when the AvrPto$_{mori}$ and AvrPto$_{lachrymans}$ homologs were expressed alone in *N. benthamiana* (data not shown). This is likely due to the endogenous Pto-like activity observed in *N. benthamiana*, which has been reported previously (He *et al.*, 2004; Yeam *et al.*, 2009). In fact, a CD loop variant of AvrPto$_{lachrymans}$ (V96A) did not elicit either Pto-mediated PCD or PCD when

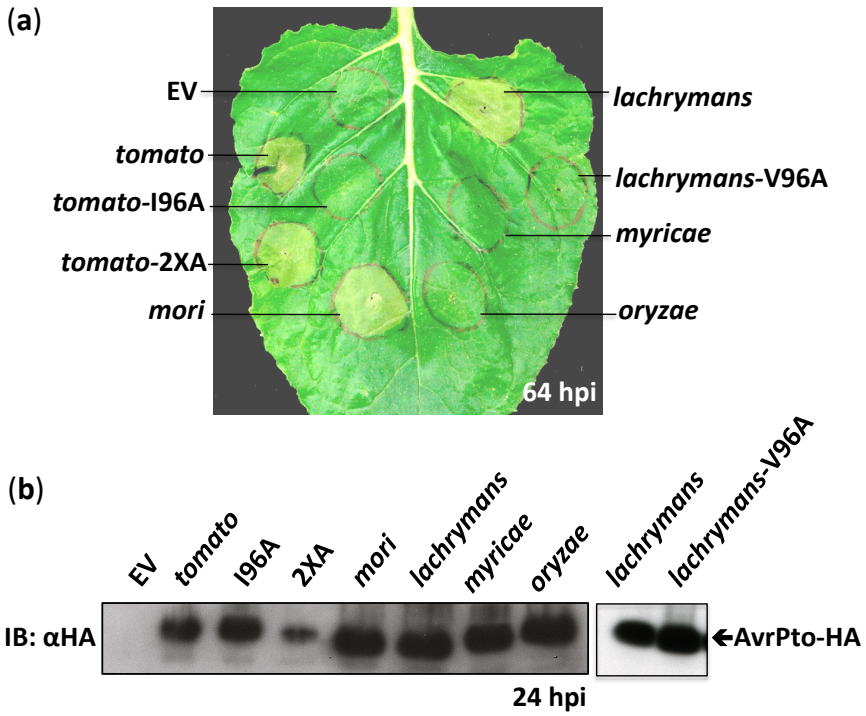

Figure 3.3. AvrPto homologs elicit a Pto-mediated hypersensitive response (HR) in *Nicotiana benthamiana*. **(A)** Pto-mediated programmed cell death (PCD) assay in *Nicotiana benthamiana* using *Agrobacterium*-mediated transient expression. HA-tagged AvrPto homologs were co-expressed with Pto in *N. benthamiana* leaves (O.D. of 0.2 each). EV indicates an empty vector control. Photographs were taken 64 hours post-inoculation (hpi). **(B)** Immunoblot using an αHA antibody was performed to detect expression of AvrPto proteins expressed in *N. benthamiana* leaves at 24 hpi.

expressed on its own in *N. benthamiana* (Figure 3.3A). Protein expression of all the AvrPto homologs in *N. benthamiana* leaves was determined by an immunoblot assay using an anti-HA antibody (Figure 3.3B).

Most AvrPto homologs promote *P. syringae* growth in susceptible plant leaves and can be recognized by either resistant tomato or tobacco

In order to compare the protein activity directly and to rule out potential discrepancies caused by minor differences at the expression level, we used the $avrPto_{tomato}$ hrp promoter to express each of the AvrPto homologs from a broad-host-range vector, pCPP45 (Lin and Martin, 2005). A FLAG-tag was fused to the C-terminus of the homologs to allow detection using anti-FLAG antibody. The protein expression and secretion of all AvrPto homologs delivered by *Pst* DC3000Δ*avrPto*Δ*avrPtoB* was confirmed (Figure 3.4A). These strains were used in the subsequent assays to further characterize the activities in *P. syringae* of the AvrPto homologs.

To examine the ability of the AvrPto homologs to promote *P. syringae* virulence, a disease assay using a low titer (10^4 cfu mL^{-1}) of *Pst* DC3000Δ*avrPto*Δ*avrPtoB* strains expressing each AvrPto homolog was performed on the susceptible tomato line Rio Grande-prf3 (RG-prf3; *Pto/Pto prf/prf*). Plants inoculated with strains expressing $AvrPto_{tomato}$, $AvrPto_{mori}$, $AvrPto_{lachrymans}$, and $AvrPto_{myricae}$ showed enhanced disease symptoms in RG-prf3 plants as compared to the empty vector plant (Figure 3.4B). Measurement of bacterial populations at 3 days post-inoculation confirmed that $AvrPto_{mori}$, $AvrPto_{lachrymans}$, and $AvrPto_{myricae}$ each enhanced bacterial growth by a statistically-significant level above the empty vector control and to a similar level as $AvrPto_{tomato}$ further supporting their virulence activity (Figure 3.4C; statistical analyses not shown).

Next, we investigated the ability of the AvrPto homologs to elicit a Pto/Prf-

dependent defense response in resistant tomato plants by performing a disease assay with the same bacterial strains on tomato Rio Grande-PtoR (RG-PtoR; *Pto/Pto Prf/Prf*). A strain containing AvrPto$_{tomato}$ showed statistically significantly decreased bacterial growth on the RG-PtoR resistant plants as compared to the empty vector control, indicating that it triggered a Pto/Prf-mediated defense (Figure 3.4C). Bacteria expressing AvrPto$_{mori}$ or AvrPto$_{lachrymans}$, also grew significantly less on RG-PtoR than on RG-prf3 plants indicating these two homologs elicit a Pto/Prf-dependent inhibition of bacterial growth. The growth data correlated with the slightly reduced disease symptoms observed in RG-PtoR plants inoculated with these two strains as compared to RG-prf3 plants (Figure 3.4B). However, since their growth on RG-PtoR was not significantly less than the empty vector control, this recognition appears to be weak. AvrPto$_{myricae}$, caused slightly enhanced bacterial growth above the empty vector control on RG-PtoR plants and no difference in bacterial growth was detected between RG-PtoR and RG-prf3 plants. These results suggest that AvrPto$_{myricae}$ has virulence activity, but does not trigger Pto/Prf-dependent inhibition of bacterial growth. Interestingly, *P. syringae* strain expressing AvrPto$_{oryzae}$ grew less than the empty vector control in both plant genotypes suggesting this homolog is recognized by a previously unknown Prf-independent resistance.

In tobacco and a related wild species, *N. sylvestris*, a putative R protein, Rpa, recognizes the CTD of AvrPto$_{tomato}$ (Yeam *et al.*, 2009). To determine if any of the AvrPto homologs are recognized by Rpa, we expressed them in *P. syringae* pv. *tabaci* and tested the ability of a low bacterial titer (10^5 cfu mL^{-1}) to elicit Rpa-mediated

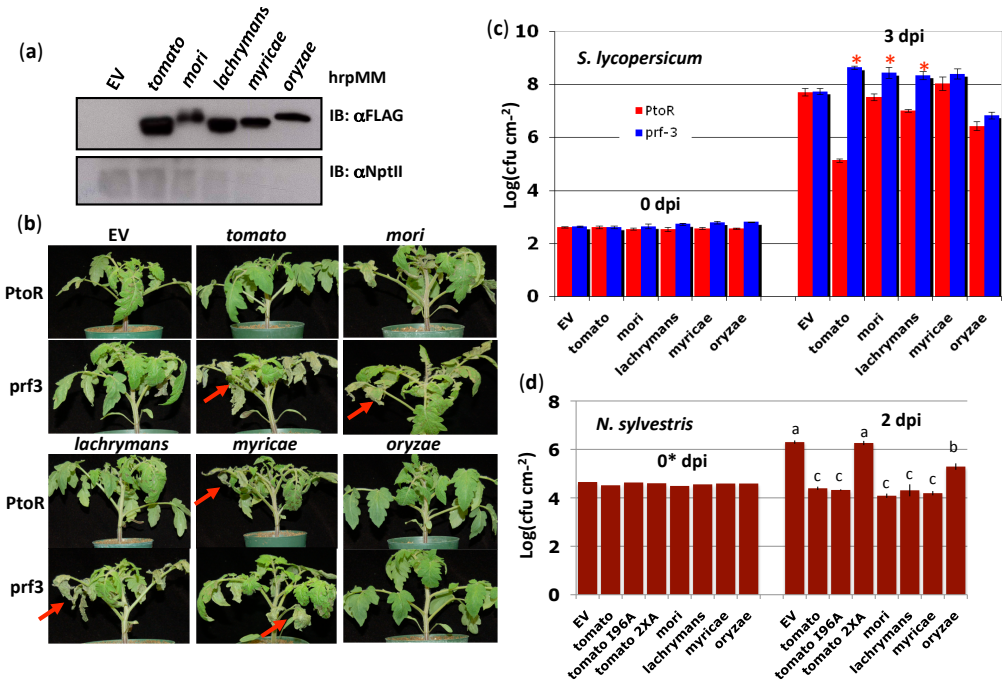

Figure 3.4. AvrPto homologs promote development of disease symptoms and bacterial growth on susceptible tomato and elicit Pto- or Rpa-mediated recognition in resistant plants. (**A**) An immunoblot assay demonstrating secretion of FLAG-tagged AvrPto proteins by *Pst* DC3000Δ*avrPto*Δ*avrPtoB*. *Pst* was grown in hrp-inducing medium and secretion of AvrPto proteins was detected with an αFLAG antibody. An αNptII antibody was used to detect cell debris contamination in the supernatant. **(B)** Disease symptoms observed on resistant tomato plants Rio Grande-PtoR (RG-PtoR; *Pto/Pto, Prf/Prf*) or susceptible tomato plants RG-prf3 (*Pto/Pto, prf/prf*) inoculated by vacuum infiltration with the *Pst* strains at a low titer (10^4 cfu/mL) at 5 days post-inoculation (dpi). EV indicates an empty vector control. Red arrows point to enhanced disease symptoms compared to the EV plants. (**C**) Bacterial population in leaves of RG-PtoR and RG-prf3 plants from the same experiment as (B) on 0 and 3 dpi. Bacterial populations were measured as colony-forming units (cfu) per square centimeter of leaf tissue. Asterisks indicate bacterial strains showing statistically significant differences in bacterial growth between RG-PtoR and RG-prf3 plants based on paired Student's t-test (P≤0.05). Errors bars indicate ± standard error (SE) (n=4). (**D**) Bacterial populations in leaves of *N. sylvestris* after inoculation with *P. s.* pv. *tabaci* strains delivering AvrPto or AvrPto variants were measured at 2 dpi. Note in this experiment, Day 0* values are log(cfu/mL) of the bacterial inoculum before infiltration. Letters above each bar represent groupings of statistical significance based on analysis of variance and comparisons for all pairs using Tukey-Kramer HSD ($P \leq 0.05$). Error bars indicate ± standard error (SE) (n=4).

resistance in *N. sylvestris*. As expected, the controls AvrPto$_{tomato}$ wild type and its CD-loop variant, AvrPto$_{tomato\ I96A}$, caused inhibition of bacterial growth while the CTD variant, AvrPto$_{tomato\ 2XA}$, did not elicit this inhibition (Figure 3.4D). AvrPto$_{mori}$, AvrPto$_{myricae}$ and AvrPto$_{lachrymans}$, but not AvrPto$_{oryzae}$, also restricted bacterial growth in *N. sylvestris* to the similar extent as wild type AvrPto$_{tomato}$. This indicates these AvrPto homologs contain functional elements that are required for the Rpa-mediated recognition in tobacco. As in tomato, the *P. syringae* strain delivering AvrPto$_{oryzae}$ grew less than the empty-vector control in tobacco, suggesting the existence of a novel recognition specificity that is both Pto/Prf- and Rpa-independent.

The CD loop and the CTD both contribute to virulence activity of certain AvrPto homologs

Residues required for virulence activity in the CD loop and the CTD are conserved in most of the AvrPto homologs based on our sequence alignment (Figure 3.2A). In AvrPto$_{tomato}$, it has been previously shown that the CD loop and the CTD both contribute to its virulence activity in an additive manner. To determine the individual contributions of these two domains to virulence activity of the homologs, substitutions were made in AvrPto$_{lachrymans}$ (CD loop and CTD) or AvrPto$_{myricae}$ (CTD only as its CD loop already lacks key residues). Each of the proteins was found to be expressed and secreted from *Pst* DC3000Δ*avrPto*Δ*avrPtoB* (Figure 3.5A). Tomato RG-prf3 plants were then inoculated with strains expressing each AvrPto homolog or its variants at a low bacterial titer (10^4 cfu mL^{-1}) for a disease assay. Consistent with previous observations, plants inoculated with bacterial strains expressing AvrPto$_{lachrymans}$ and AvrPto$_{myricae}$ showed more severe disease symptoms than the empty vector. However, we observed a reduction in disease severity in plants infected with strains expressing the CD loop or CTD variants of AvrPto$_{lachrymans}$ and

AvrPto$_{myricae}$ (Figure 3.5B). In the bacterial population assay, as with AvrPto$_{tomato}$, substitutions in the CD loop or the CTD of AvrPto$_{lachrymans}$ reduced its virulence activity as compared to wild type but not to the level of the empty vector control strain (Figure 3.5B and C). In the case of AvrPto$_{myricae}$, we observed a reduction in disease symptoms on plants infected with the bacterial strain expressing its CTD variant, AvrPto$_{myricae\ 2XA}$, as compared to the wild type although a statistically significant difference was not detected in bacterial growth (Figure 3.5B and C). Together, these results suggest that the functions of the two virulence determinants are conserved in certain AvrPto homologs implying their virulence targets in their respective hosts might be similar to those in tomato.

It has been shown in both Arabidopsis and tomato that the CD loop structure of AvrPto promotes virulence by disrupting PRR complexes and blocking downstream signaling pathways, leading to suppression of PAMP-induced MAPK activation (He *et al.*, 2006). The CTD was shown recently to promote virulence in a manner that is mechanistically distinct from the CD loop (Yeam *et al.*, 2009). To examine whether the mechanisms by which the AvrPto homologs promote virulence are conserved, we assessed the ability of AvrPto homologs and variants to suppress flg22-induced MAPK activation in tomato (RG-prf3) protoplasts (Figure 3.5D). AvrPto$_{lachrymans}$, as with AvrPto$_{tomato}$, was capable of suppressing flg22-induced MAPK activity. Neither AvrPto$_{myricae}$ or AvrPto$_{oryzae}$ suppressed MAPK activation, which was as expected since both of them lack key residues required for a functional CD loop structure. We also found that the CD loop but not the CTD of AvrPto$_{lacrymans}$ was responsible for its ability to suppress MAPK activation triggered by flg22 treatment, which is consistent with our previous results with AvrPto$_{tomato}$ (Yeam *et al.*, 2009). These data further support a role of the CD loop structure in suppressing PTI and the CTD in promoting virulence by a distinct, unknown, mechanism.

Unexpectedly, AvrPto$_{mori}$ did not suppress MAPK activation although it interacted with the Pto kinase in the yeast two-hybrid assay, caused PCD when co-expressed with Pto in *N. benthamiana*, and triggered a Pto/Prf-dependent inhibition of the bacterial growth in tomato. This may be due to the fact that although AvrPto$_{mori}$ has several key residues required for the CD loop function, there are four other polymorphisms in the CD loop of this protein as compared to AvrPto$_{tomato}$ (Figure 3.2A). It is therefore possible these polymorphisms affect the ability of AvrPto$_{mori}$ to suppress certain PTI pathways associated with MAPK activation.

The CD loop and the CTD of the AvrPto homologs elicit Pto-mediated resistance and Rpa-mediated resistance, respectively.

Previous studies showed that Pto-mediated recognition of AvrPto is dependent on the CD loop structure (Chang *et al.,* 2001; Wulf *et al*., 2006; Xing *et al*., 2007). We observed a weak Pto/Prf-dependent recognition of AvrPto$_{mori}$ and AvrPto$_{lachrymans}$ (Figure 3.4C). To determine whether this recognition is due to the CD loop, we used *Pst* DC3000Δ*avrPto*Δ*avrPtoB* strains expressing the AvrPto homologs or their variants for a disease assay in tomato RG-PtoR. We observed enhanced growth compared to the wildtype protein when a V96A substitution was introduced into the CD loop of AvrPto$_{lachrymans}$ (AvrPto$_{lachrymans\ V96A}$) (Figure 3.6A). This indicates the growth difference of *Pst* strain expressing AvrPto$_{lachrymans}$ observed between RG-prf3 and RG-PtoR is most likely due to a Pto/Prf-mediated recognition. However, as also observed in Figure 3.4C, we were not able to detect significant difference in bacterial growth between strains expressing AvrPto$_{lachrymans}$ and the empty vector implying that this recognition is weak. In the case of AvrPto$_{myricae}$, which naturally lacks key residues in the CD loop, it showed elevated bacterial growth level compared to the empty vector control, indicating that AvrPto$_{myricae}$ is not recognized by Pto/Prf

complex consistent with our earlier observation in Figure 3.4C. The substitutions in the CTD for AvrPto$_{lachrymans\ 2XA}$ and AvrPto$_{myricae\ 2XA}$ did not cause significantly increased bacterial growth on RG-PtoR plants compared to their wild types, which is also consistent with the previous results with AvrPto$_{tomato}$ (Anderson *et al.*, 2006).

We showed previously in Figure 3.4D that AvrPto$_{mori}$, AvrPto$_{lachrymans}$, and AvrPto$_{myricae}$, which have the two conserved serine residues (S147 and S149) in the CTD, elicit strong Rpa-mediated resistance in *N. sylvestris*. In order to determine whether the CTD of these AvrPto homologs is responsible for Rpa-mediated recognition we performed a disease assay using *P. syringae* pv. *tabaci* strains expressing each AvrPto homolog and its CTD variants (Figure 3.6B and C). Substitutions in the CTD of AvrPto$_{lachrymans}$, AvrPto$_{myricae}$, and the AvrPto$_{tomato}$ control completely abolished their recognition, as is indicated by the increased bacterial growth they allowed in comparison with their wild type proteins (Figure 3.6B). Consistent with the bacterial growth assay, disease-associated cell death was only observed in leaf areas inoculated with strains expressing the empty vector or AvrPto homologs with Ser to Ala substitutions in their CTD (Figure 3.6C). This indicates that the avirulence activity of the CTD is also conserved in AvrPto homologs.

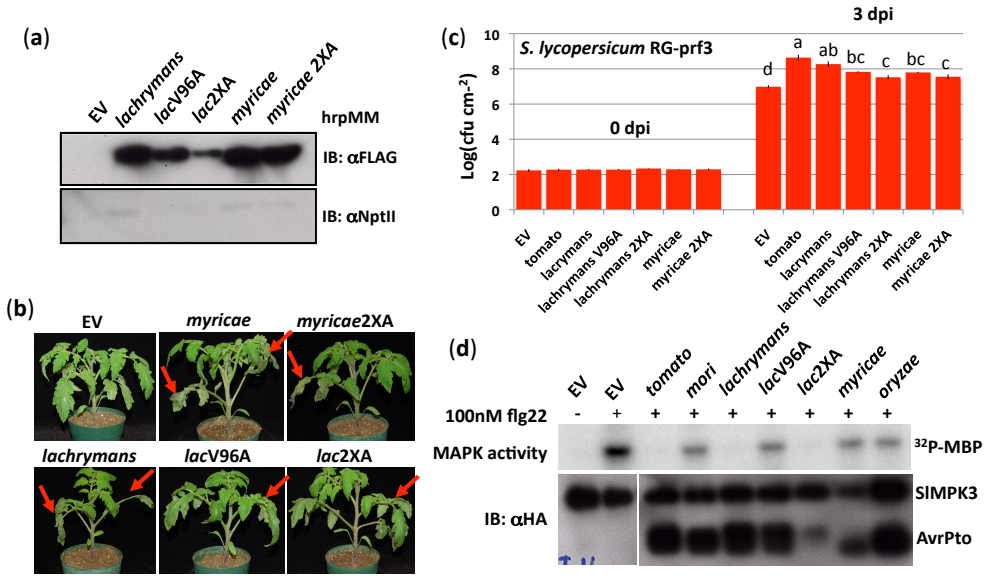

Figure 3.5. Virulence activities of the CD loop and the CTD are conserved in AvrPto homologs. (**A**) Immunoblot to examine secretion from *Pst* DC3000Δ*avrPto*Δ*avrPtoB* of FLAG-tagged AvrPto proteins. *Pst* was grown in hrp-inducing medium and secretion of AvrPto proteins was detected with an αFLAG antibody. An αNptII antibody was used to detect cell debris contamination in the supernatant. (**B**) Disease symptoms observed on susceptible tomato plants RG-prf3 (*Pto/Pto prf/prf*) inoculated with *Pst* DC3000 Δ*avrPto*Δ*avrPtoB* strains delivering AvrPto proteins (10^4 cfu/mL) at 5 dpi. Red arrows indicate enhanced disease symptoms as compared to the empty vector (EV) control. (**C**) Bacterial population assays in RG-prf3 plants from the same experiment as (b) at 0 and 3 dpi. Letters above each bar represent groupings of statistical significance based on analysis of variance and comparisons for all pairs using Tukey-Kramer HSD ($P \leq 0.05$). Error bars indicate ± standard error (SE) (n=4). (**D**) An immunoblot assay to determine ability of AvrPto proteins to suppress flg22-induced activation of tomato MAPK (SlMPK3) in tomato protoplasts. HA-tagged AvrPto proteins were expressed with HA-tagged SlMPK3 in tomato RG-prf3 protoplasts. Transfected protoplasts were treated with 100 nM flg22 for 10 minutes. An αHA antibody was used to immunoprecipitate SlMPK3 and AvrPto proteins. Upper panel show an *in vitro* assay detecting SlMPK3 phosphorylation of myelin basic protein (MBP; upper panel). Lower panel is a protein gel blot showing expression of SlMPK3-HA and AvrPto-HA using αHA antibody.

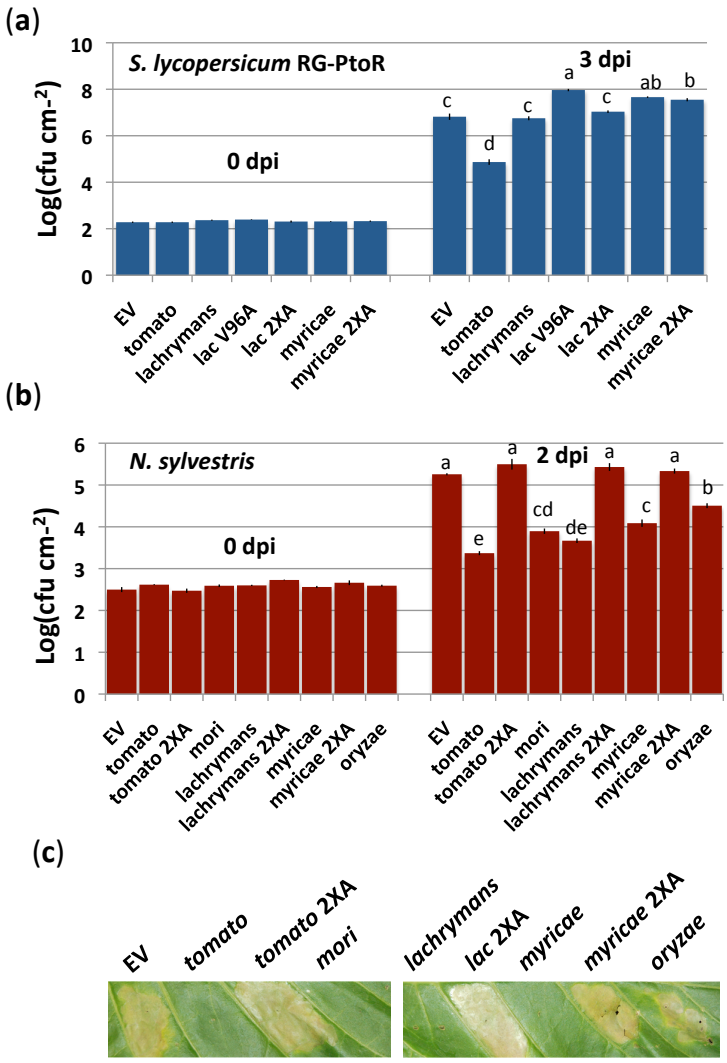

Figure 3.6. Avirulence activities of the CD loop and CTD are conserved. (**a**) Bacterial population assays on RG-PtoR plants inoculated with the same bacterial strains as in Figure 5A at 0 and 3 dpi. Letters above each bar represent groupings of statistical significance based on analysis of variance and comparisons for all pairs using Tukey-Kramer HSD ($P \leq 0.05$). Error bars indicate ± standard error (SE) (n=4). (**b**) Bacterial populations were measured in leaves of *Nicotiana sylvestris* after inoculation with *P. s.* pv. *tabaci* strains delivering the AvrPto proteins indicated (10^5 cfu/mL) at 0 and 2 dpi. (**c**) Disease symptoms observed on *N. sylvestris* leaves from the same experiment as (b) at 6 dpi.

Discussion

Type III effector AvrPto is one of the few T3Es that have been shown to contribute significantly to the fitness of *Pst* (Cunnac *et al.*, 2009). Previous studies reported the presence of *avrPto*-related sequence in many *P. syringae* pathovars infecting a wide range of host plants (Sarkar et al., 2006; Lin and Martin, 2007). It has been suggested that this effector may promote virulence and elicit host recognition in diverse *P. syringae* pathovars in which it occurs (Lin and Martin, 2007). In this study, we found that three of the four sequence-diverse AvrPto homologs we examined do in fact have virulence activity and are able to be recognized by species in two different Solanaceae genera. Further characterization of the two functional domains of AvrPto in some homologs indicated that their virulence-promoting mechanisms are also conserved. Our present data, however, leave unanswered the question of whether any of these AvrPto homologs promote virulence or trigger resistance when delivered from their native strain into their host plant species.

AvrPto undergoes several host-mediated post-translational modifications once delivered into the host cell cytoplasm and has been shown to have a modular structure with discrete domains displaying distinct virulence activities (Shan *et al.*, 2000b; Anderson *et al.*, 2006; Yeam *et al.*, 2009). Myristoylation of the N-terminal glycine of AvrPto, which targets it to the plant plasma membrane (PM), is required for all AvrPto activities and indicates that key host targets for this effector are present at the PM (Shan *et al.*, 2000b). Another domain, the CD loop, disrupts host receptor kinase complexes localized to the PM and hence blocks their downstream signaling pathways (Shan *et al.*, 2008). Finally, the CTD is phosphorylated by an unknown host kinase, and this modification promotes AvrPto virulence (Anderson *et al.*, 2006). The phosphorylated CTD contributes additively to AvrPto virulence via a distinct mechanism from that of the CD loop although its virulence mechanism is unknown

(Yeam *et al.*, 2009). Interestingly, both virulence domains are monitored by host recognition mechanisms (Yeam *et al.*, 2009). Here we have shown that, despite the occurrence of many polymorphisms throughout their coding sequence, the previously identified virulence domains are functionally conserved in AvrPto homologs from diverse *P. syringae* pathovars. PAML analysis was performed on these sequences but it did not support the occurrence of positive selection on any residue (data not shown). However, the fact that AvrPto homologs have undergone considerable diversification in their DNA sequences, but that key virulence residues are conserved, suggests there may have been pressure to maintain these domains – possibly due to the widespread conservation of the host processes they are targeting in diverse plant species.

In recent years, it has become evident that one of the main virulence functions of pathogen effectors is to suppress the first line of host defense, PTI (Zhou and Chai, 2008). The ability of ~20 *Pst* DC3000 effectors to suppress one or more PTI-related processes has been demonstrated in several studies (Cunnac *et al.*, 2009). Interestingly, while a few effectors are effective in one PTI suppression assay but ineffective in others, AvrPto (and AvrPtoB) are capable of suppressing PTI in all assays in which they have been examined so far suggesting these effectors act on early events in host PTI and play a central role in bacterial pathogenesis (Cunnac et al., 2009). AvrPto$_{lachrymans}$ suppressed flg22-induced MAPK activation, suggesting that this effector might target FLS2/BAK1 and possibly other PRR complexes in cucumber – the natural host of *P. s.* pv. *lachrymans*. The fact that suppression of flg22-induced MAPK activation by AvrPto$_{lachrymans}$ was dependent on its CD loop structure and not its CTD further supported our previous observation that the CTD promotes virulence activity via a different mechanism from that of the CD loop (Yeam *et al.*, 2009).

Interestingly, AvrPto$_{mori}$ did not suppress MAPK activation induced by flg22 in the same assay despite its ability to interact with Pto in yeast and trigger PCD in *planta*

(Figure 3.5C). There are 4 polymorphisms within the CD loop of AvrPto$_{mori}$ as compared to AvrPto$_{tomato}$ including E93, R95, V96, and R101. Previously, V96 and R101 have been found to not affect Pto interaction and virulence activity of AvrPto$_{tomato}$ (Pascuzzi, 2006). Pto was suggested to be a 'molecular mimic' of the virulence targets of AvrPto's CD loop since mutations that abolish the interaction between the two also abolish the CD loop's ability to suppress PTI (He *et al*., 2006; Xiang *et al*., 2008). It is possible that one of the polymorphisms present in the CD loop of AvrPto$_{mori}$ uncouples these two functions; however, that hypothesis remains to be tested.

The two virulence domains of AvrPto, the CD loop and the CTD are monitored by two distinct host recognition mechanisms (Yeam *et al.*, 2009). Several examples from other *P. syringae* effectors, including AvrRpt2, AvrPtoB and the HopZ1 family indicate that host recognition usually monitors the domain of an effector that is important for its virulence activity (Chisholm *et al.*, 2005; Lin *et al.*, 2006; Xiao *et al.*, 2007a; Xiao *et al.*, 2007b; Zhou *et al.*, 2009). This likely creates strong selection pressure on the effector gene to evolve to escape this recognition while maintaining its virulence (Ma *et al.*, 2006). A well-characterized example of the co-evolutionary arms race between an effector and its host recognition is the relationship between HopZ1 and its corresponding plant R gene. Driven by the strong selective pressure from the host, the ancestral form, HopZ1a, has been replaced by mutational derivatives allowing the bacterial to escape the host recognition. Interestingly, these modified versions of HopZ1a maintain the cysteine protease activity, which is also involved in triggering resistance response in host plant carrying the cognate resistance protein (Ma *et al.*, 2006; Zhou *et al.*, 2009). Although the molecular mechanism by which HopZ1 alleles evolved to escape host recognition and maintain virulence activity is unknown, phytoadaptation seems to be an important mechanism that pathogen effectors employ

under strong selective pressure from the host defense systems.

Clearly, it is too early to draw conclusions about the co-evolutionary relationships among the AvrPto homologs and their host recognition mechanisms. Nevertheless, it is intriguing to consider the role that multiple virulence domains, as observed in AvrPto, might play as a common feature in type III effectors (Yeam *et al.*, 2009). Perhaps one of the evolutionary advantages of such modularity is to lessen the cost of escaping host recognition by allowing the retention of partial virulence activity. In this regard, it is interesting that AvrPto$_{myricae}$ has a CD loop that is not recognized by Pto but also lacks virulence activity; the protein however retains a virulence-promoting CTD. It is possible this is an example of a natural variant that has evolved to escape a Pto-like recognition by altering its CD loop. With the sequence variation we report here as a guide, it will be interesting to examine AvrPto from *P. syringae* found in tomato fields where Pto is commonly used to examine whether the effector might have evolved to alter its CD loop while retaining its CTD virulence function.

AvrPto$_{oryzae}$ shares only 47% amino acid identity with AvrPto$_{tomato}$ and neither its CD loop or its CTD were functional in promoting virulence, or indeed, in being recognized by Pto or Rpa. Surprisingly, expression of AvrPto$_{oryzae}$ in *Pst* appeared to elicit a Pto/Prf- and Rpa-independent host defense, suggesting this protein is recognized by a novel resistance protein. It is important to point out, however, that we were unable to detect expression in the *P. s.* pv. *oryzae* strain of either the RNA or protein corresponding to AvrPto$_{oryzae.}$ It is possible therefore that AvrPto$_{oryzae}$ is simply not expressed in this strain despite the fact that it has an intact open reading frame. This might be due to its recent horizontal transfer and the lack of a functional promoter or to some other mechanism that acts to suppress expression certain effectors. We have reported an example of apparent post-transcriptional regulation previously in which AvrPtoB is expressed in a *Pst* strain at the RNA level although its

protein is undetectable (Lin *et al.*, 2006).

The recently sequenced genome of *P. s.* pv. *tabaci* (*Pta*) revealed a distant homolog of AvrPto (41% amino acid identity with AvrPto$_{tomato}$) that is reported to not be recognized by the Pto/Prf resistance protein complex (Studholme *et al.*, 2009). Although we did not examine AvrPto$_{tabaci}$ here, a previous study showed that this homolog was not detected by DNA hybridization using *avrPto*$_{tomato}$ as a probe (Lin and Martin, 2007). This homolog is highly divergent from AvrPto$_{tomato}$ and lacks key residues in the CD loop (Figure 3.2A), that would explain its inability to elicit Pto/Prf-mediated defense (Studholme *et al.*, 2009). It has been shown previously and in this study that *Pta* itself does not elicit either Pto-mediated or Rpa-mediated defense responses in *Nicotiana* species (Yeam *et al.*, 2009). It is possible therefore that AvrPto$_{tabaci}$ has undergone diversifying selection to avoid host recognition. Whether or not AvrPto$_{tabaci}$ or AvrPto$_{oryzae}$ have virulence activity in their respective compatible host plants remains to be tested.

We observed that Rpa-mediated recognition of homologs with the conserved CTD seemed to be more robust than Pto-mediated recognition of homologs with the conserved CD loop. For instance, there was a mild indication of disease in tomato expressing Pto when it was infected with *Pst* strains delivering AvrPto$_{lachrymans}$, which has both a conserved CD loop and CTD. In contrast, in tobacco, Rpa-mediated recognition of AvrPto$_{lachrymans}$ is as strong as that of AvrPto$_{tomato}$. These results suggest that the eventual cloning of the *Rpa* gene may provide an alternative and robust form of resistance against *P. syringae* containing AvrPto homologs that might have evolved to escape Pto recognition of the CD loop. In the future, it will be interesting to study the possible correlation between the host range of *P. syringae* strains expressing AvrPto, the conservation of the CD loop and/or the CTD in these strains, and the ability of the hosts to recognize these two domains.

Materials and Methods

Cloning and characterization of *avrPto* homologs

Genomic DNA (2μg) isolated from bacteria grown in rich media and digested with *HindIII*, *BamHI*, and *ClaI* was used for DNA gel blot analysis following procedures described previously (Fouts et al., 2002; Lin and Martin, 2005). PCR using *avrPto-*$_{tomato}$-specific primers was performed to obtain *avrPto* homologs from *P. syringae* pathovars *tomato, maculicola*, and *syringae* (Table 3.1). To isolate the other *avrPto* sequences, genomic libraries were prepared from pathovars *mori, lachrymans, papulans, myricae,* and *oryzae*, in the vector pBlueScript (Stratagene, Cedar Creek, TX) and screened by colony hybridization with an *avrPto*$_{tomato}$ fragment. Bacterial genomic DNA fragments were cloned into pBlueScript (Stratagene, Cedar Creek, TX) and the ligation products were transformed into *Escherichia coli* (DH5α). The transformed colonies were used for colony blot hybridization. GenBank numbers for the AvrPto homologs are as follows: GQ227720 (AvrPto$_{myricae}$), GQ227721 (AvrPto$_{mori}$), GQ227722 (AvrPto$_{lachrymans}$), and GQ227723 (AvrPto$_{oryzae}$). Sequence alignment and phylogenetic analyses were performed using MegAlign (DNASTAR Inc., Madison, WI).

Preparation of bacterial cells for RNA and protein assays, and RNA extraction and cDNAs synthesis were done following protocols described previously (Lin *et al.*, 2006). Gene-specific primers for each *avrPto* homolog (Table 3.3) were used in RT-PCR to determine their expression at the RNA level. A primer set to amplify 23S rRNA from cDNAs was used to check the quality of the cDNA as an internal control. Expression of AvrPto proteins were detected with an αAvrPto$_{tomato}$ antibody using standard immunoblotting protocols (Sambrook *et al.*, 1989).

A broad host range vector pCPP45 with the *avrPto*$_{tomato}$ hrp-inducible promoter was used to compare the activities of the AvrPto homologs with each having a FLAG tag fused at the C-terminus. The constructs were transformed into *Pst* DC3000Δ*avrPto*Δ*avrPtoB* or *Pta* 11528R by electroporation. Expression and secretion levels of each AvrPto protein by *P. s.* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* were examined by immunoblot assays using αFLAG antibodies following protocols described previously (Lin *et al.*, 2006).

***Agrobacterium*-mediated transient expression**

Agrobacterium tumefaciens strain GV2260 was used for transient gene expression in four-week old *N. benthamiana* leaves. All AvrPto homologs with a C-terminal HA epitope tag were expressed under the CaMV 35S promoter from pLN462 (Jamir *et al.*, 2004). Pto was expressed under the CaMV 35S promoter from pBTEX. Presence or absence of cell death caused by co-expressing AvrPto with Pto or AvrPto alone (data not shown) was determined at 64 hours post inoculation (hpi). Data presented represent a minimum of three independent experiments. Expression of AvrPto proteins was confirmed in *N. benthamiana* 24 hpi, prior to visible cell-death appearance. The antibody used for immunoblot assays was αHA (Roche Applied Science, Indianapolis, IN).

Table 3.2. Full list of *P. syringae* strains used in this study, their putative hosts, and their sources. Strains that have *avrPto*-like sequence are indicated.

Pathovars	Strains	Hosts	Presence of *avrPto*	Source
actinidiae	FTRS_L1	Kiwi		Guttman[1]
antirrhini	PDDCC4303	Snapdragon		Martin
atrofaciens	B143	Wheat		Martin
coronofaciens	KN221	Oat	yes	Martin
delphinii	PDDCC529	Delphinium		Martin
glycinea	R4a	Soybean	yes	Guttman
glycinea	KN28	Soybean		Guttman
glycinea	KN44	Soybean		Guttman
glycinea	LN10	Soybean		Guttman
glycinea	MOC601	Soybean		Guttman
glycinea	race 6	Soybean		R. Innes[2]
glycinea	race 5	Soybean		R. Innes
glycinea	2159 race 1	Soybean		Guttman
lachrymans	N7512	Cucumber	yes*	Guttman
lachrymans	YM7902	Cucumber	yes	Guttman
lachrymans	YM8003	Cucumber	yes*	Guttman
lachrymans	107	Cucumber	yes*	Guttman
maculicola	M6	Cauliflower		Guttman
maculicola	4981	Cauliflower	yes*	Guttman
maculicola	KN203	Chinese cabbage	yes*	Guttman
maculicola	KN91	Radish		Guttman
maculicola	KN84	Radish		Guttman
maculicola	ES4326	Radish		Guttman
maculicola	YM7930	Radish		Guttman
mellea	N6801	Tobacco		Guttman
mori	MAFF301020	Mulberry	yes*	Guttman
mori	PDDCC4331	Mulberry	yes*	Guttman
morsprunum	PDDCC4352	Japanese apricot		Martin
myricae	MAFF302941	Bayberry	yes*	Guttman
myricae	AZ84488	Bayberry	yes*	Guttman
oryzae	36_1	Rice	yes*	Guttman
oryzae	I_6	Rice	yes*	Guttman
persicae	PDDCC5846	Nectarine		Martin
phaseolicola	1302A	Kidney bean		Guttman
phaseolicola	SG44	Kidney bean		Guttman

Table 3.2 (Continued):

phaseolicola	Y5_2	Kudzu		Guttman
phaseolicola	HB10Y	Kidney bean		Guttman
phaseolicola	NS368	Kidney bean		Guttman
phaseolicola	KN86	Kidney bean		Guttman
phaseolicola	1449B	Kidney bean		Guttman
phaseolicola	PP14	Kidney bean		P. Lindgren[3]
phaseolicola	NPS3121	Kidney bean		P. Lindgren
phaseolicola	NPS4000	Kidney bean		P. Lindgren
pisi	H5E3	Pea		Guttman
pisi	PP1	Pea		Guttman
pisi	895A	Pea		Guttman
pisi	H6E5	Pea		Guttman
papulans	5	Apple		Martin
sesami	HC_1	Sesame		Guttman
solanacearum	UW8 race 1	Solanaceous plants		Martin
solanacearum	UW25 race1	Solanaceous plants		Martin
solanacearum	UW275 race 1	Solanaceous plants		Martin
syringae	1212R	Pea	yes*	Guttman
syringae	B728A	Snap bean	yes*	Guttman
syringae	Ps9220	Spring onion	yes*	Guttman
syringae	A2	Ornamental pear		Guttman
syringae	B64	Wheat		Guttman
syringae	L177	Lilac		Guttman
tagetis	BK376	Common ragweed		Martin
tomato	DC3000	Tomato	yes*	Martin
tomato	DCT6D1	Tomato	yes*	Guttman
tomato	DC84_1	Tomato	yes*	Guttman
tomato	DC89_4H	Tomato	yes*	Guttman
tomato	Bakerfield	Tomato	yes*	J. Watterson
tomato	PT11	Tomato	yes*	L. Walling [4]
tomato	PT12	Tomato		L. Walling
tomato	PT23	Tomato	yes*	L. Walling
tomato	133	Tomato	yes*	Martin
tomato	1108	Tomato	yes*	D. Cupples
tomato	NYS race 0	Tomato	yes*	C. Smart[5]
tomato	407	Tomato		C. Smart
tomato	409	Tomato	yes*	C. Smart
tomato	303	Tomato		C. Smart
tomato	A1	Tomato	yes*	Martin

Table 3.2 (Continued):

tomato	A9	Tomato		Martin
tomato	Gilreath #4	Tomato	yes*	J. Jones
tomato	Gilreath #6	Tomato	yes*	J. Jones
tomato	J.Scott	Tomato	yes*	J. Jones

Note:

*These AvrPto homologs were cloned and sequence-verified.

[1] Dr. D. Guttman (University of Torronto) (Sakar and Guttman, 1999)
[2] R. Innes (University of Indiana)
[3] P. Lindgren (North Carolina State)
[4] L. Walling (University of California, Riverside)
[5] C. Smart (Cornell University)

Table 3.3. Primers used in this study.

Primer name	Sequence (5' → 3')
AvrPtoF	CACCATGGGAAATATATGTGTCGG
AvrPtoR	TTGCCAGTTACGGTACGGGCTA
moriF	CACCATGGGAAATATTTGTGTCGGCG
moriR	CCAGTTCCTGTAAGGGCTCAGG
lacF	CACCATGGGAAATATATGTGTTGGCG
lacR	GTTTCCCCAAGGCCTTGGAGAA
myricaeF	CACCATGGGAAACATATGTGTCGG
myricaeR	GATCAGCTTTCCCAAGGTCTAG
oryzaeF	CACCATGGGAAACATCTGTGTAGGCG
oryzaeR	CCGATTTCCGTAAGGGCTAGGT
lac**V96A**F*	GAGCTTCAGGTGCCAATCCTGGAAT
lac**V96A**R*	ATTCCAGGATTGGCACCTGAAGCTC
lac**2XA**F*	CTATAAATCCGGCTGGGGCAATCAGAATGGCG
lac**2XA**R*	CGCCATTCTGATTGCCCCAGCCGGATTTATAG
myri**2XA**F*	ACTATTAATCCAGCTGGAGCAATTCGAATGGC
myri**2XA**R*	GCCATTCGAATTGCTCCAGCTGGATTAATAGT

*Amino acid substitutions were made using PCR-based (Pfu turbo tag polymerase) side-directed mutagenesis reactions with these primer pairs (amino acid changes in bold).

Protoplast assays for MAP kinase suppression

Three- to four-week-old tomato Rio Grande-prf3 (*Pto/Pto prf/prf*) leaves were used for protoplast isolation (Rosebrock *et al.*, 2007). Protoplasts were co-transformed with a vector expressing the HA-tagged SlMPK3 and HA-tagged AvrPto homologs using a polyethylene glycol protocol described previously (Xiao *et al.*, 2007). Transfected protoplasts were incubated for 6 hours and then treated with 100nM flg22 for 10 minutes. Detection of the AvrPto-mediated suppression of flg22-induced MAPK activity was performed as described previously (He *et al.*, 2006). Data presented are representative of a minimum of three independent experiments.

Pathogenesis assays in tomato and tobacco

Five- to six-week-old plants of tomato Rio Grande-prf3 (*Pto*/*Pto prf*/*prf*) or Rio Grande-PtoR (*Pto/Pto Prf/Prf*) were vacuum-infiltrated with the different *Pst* DC3000 strains at an inoculum level of 10^4 colony-forming units mL^{-1} (cfu mL^{-1}) and maintained in a climate-controlled growth chamber as described previously (Anderson *et al.*, 2006). Four- to five-week-old *Nicotiana sylvestris* plants were used for inoculating different *Pta* 11528R strains (10^5 cfu mL^{-1}) by syringe infiltration. Bacterial enumeration and statistical analysis were performed as described previously (Yeam *et al.*, 2009). Data presented represent a minimum of three independent experiments.

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CHAPTER 4

HOST-MEDIATED PHOSPHORYLATION OF THE C-TERMINAL DOMAIN OF *PSEUDOMONAS SYRINAGE* EFFECTOR AVRPTO IS Ca^{2+}-DEPENDENT

Abstract

The type III effector protein AvrPto from *Pseudomonas syringae* pv. *tomato* (*Pst*) is secreted into plant cells during bacterial infection of tomato leaves. It is one of the few effectors that contribute demonstrably to *Pst* virulence in susceptible plants. AvrPto has two virulence determinants, the CD loop and the phosphorylated C-terminal domain (CTD), that contribute additively to its virulence activity. The CD loop targets pathogen associated molecular pattern (PAMP) receptor complexes to disrupt PAMP-triggered immunity (PTI) while the CTD promotes bacterial virulence via a different and yet unknown mechanism. Interestingly, the two virulence determinants are monitored by two distinct recognition mechanisms found in the Solanaceae family: the CD loop is recognized by tomato Pto/Prf while the phosphorylated CTD is recognized by Rpa, which has only been observed in certain *Nicotiana* species. We show here that the kinase activity phosphorylating AvrPto's CTD, termed Avk for AvrPto kinase, is induced by PAMPs, indicating that this kinase(s) might be involved in PTI. Using Arabidopsis functional protein microarrays, we screened for candidate kinases and identified calcium-dependent protein kinases (CDPKs) as potential Avks. In addition, we found that Avk activity *in vitro* is Ca^{2+}-dependent, which supports a role of CDPKs as Avks.

Introduction

Many Gram-negative bacteria employ the type III secretion system (T3SS) to secrete a suite of effectors into plant cells during the infection process (Alfano and Collmer, 2004). One of the main activities of most bacterial effectors, despite their diversity, is to suppress host-induced defense responses to promote bacterial pathogenesis (Abramovitch and Martin, 2004; Cunnac et al., 2009). Upon sensing the presence of pathogen-associated molecular patterns (PAMPs), the host plant pattern-recognition receptors (PRRs) trigger a cascade of signaling pathways which results in plant responses such as the production of reactive oxygen species (ROS), callose deposition at the plant cell wall, and a suite of cellular responses that halt the pathogen infection (Boller and Felix, 2009). Therefore, it is essential for a successful bacterium to be able to subvert this first line of plant defense via the actions of its T3SS effectors (T3Es). Plants have also evolved strategies to monitor the presence of these effectors and trigger a stronger defense response, termed effector-triggered immunity (ETI). ETI is usually mediated through 'gene-for-gene' recognition, in which the product of a plant resistance gene directly or indirectly detects the presence or action of a bacterial effector to elicit the hallmark hypersensitive response, or programmed cell death to render the plant resistant to bacterial pathogens carrying that effector (Flor, 1955; Chisholm et al., 2006). The complex interaction between the plant host and its bacterial pathogen can be viewed as a co-evolutionary 'arms race' in which each organism strives to survive.

Several bacterial effectors have evolved to be specific substrates for certain plant enzymes leading to host-mediated post-translational modifications of the effectors upon being delivered into the host cells. These modifications include acylation, ubiquitination, phosphorylation, and proteolytical cleavage. Detailed functional studies have revealed that host-mediated modifications are important for the

virulence activity of some effectors and that their modification status is often monitored by the host surveillance mechanism. For example, N-terminal myristoylation is required proper membrane localization for host recognition of *Pseudomonas syringae* effectors AvrPto, AvrB, AvrRpm1, AvrPphB, and HopZ effector family and for the virulence functions of AvrPto, AvrRpm1, and HopZ (Nimchuk et al., 2000; Shan et al., 2000; Lewis et al., 2008). Plasma membrane localization is consistent with the role of some effectors in targeting transmembrane PRRs and their associated signaling pathways to suppress PTI (He et al., 2006; Shan et al., 2008). AvrRpt2 is a cysteine protease that is self-cleaved in *planta* into a stable 21-kDa protein, which is sufficient for its activity such as cleaving RIN4 (Mudgett and Staskawicz, 1999). Prior to cleaving its N-terminus, AvrRpt2 has to be activated by the host cyclophilin, a conserved eukaryotic peptidyl-prolyl isomerase, for proper protein folding (Coaker et al., 2005). It has been suggested that host-mediated activation of bacterial effectors is a common theme in bacterial pathogenesis as enzymatic activities of many effectors cannot be detected *in vitro* (Coaker et al., 2005).

Another widespread host-mediated modification of bacterial effectors is phosphorylation by a host kinase activity. Examples of such effectors include *P. syringae* effectors AvrPto, AvrPtoB, AvrB, and *Rhizobium* sp. NGR234 effectors NopL and NopP (Bartsev et al., 2003; Skorpil et al., 2005; Anderson et al., 2006; Desveaux et al., 2007; Xiao et al., 2007). While phosphorylation has been implicated to be important for the effector activities, the role of this post-translational modification in enhancing bacterial virulence and eliciting host recognition has only been experimentally demonstrated for AvrPto and AvrPtoB (Anderson et al., 2006; Xiao et al., 2007).

P. syringae pv. *tomato* (*Pst*), the causative agent of bacterial speck disease in tomato, delivers about 30 effectors into host cells during its infection process (Cunnac et al., 2009). Among them, AvrPto is the best studied. It is one of the few effectors that have been shown to have a significant effect on bacterial growth and disease symptoms in susceptible plants (Lin and Martin, 2005; Kvitko et al., 2009). AvrPto is a small (18 kDa) hyprophilic protein that has multiple domains that are important for its function (Ronald et al., 1992). Once delivered into the host cell, AvrPto is myristoylated and targeted to the plasma membrane, which is absolutely required for its activity (Shan et al., 2000).

AvrPto has two functional domains that contribute additively to its virulence, and are monitored by distinctive host recognition mechanisms: the CD loop and the phosphorylated C-terminal domain (CTD) (Pascuzzi, 2006; Yeam et al., 2010). The CD loop is responsible for AvrPto ability to suppress PTI, which involves binding and inhibiting kinase activity or disrupting complex formation of membrane-associated PRRs such as FLS2, EFR, and BAK1 (Shan et al., 2008; Xiang et al., 2008). The tomato resistance protein complex Pto/Prf, in which Pto is a Ser/Thr kinase and Prf is a classic nucleotide-binding leucine rich repeat protein (NB-LRR), recognizes the CD loop domain via the direct interaction of Pto and AvrPto (Salmeron et al., 1996; Tang et al., 1996; Mucyn et al., 2006). This interaction has been proposed to promote conformational changes in Pto, which is detected by Prf and thereby triggers ETI (Xing et al., 2007). The Pto kinase domain is similar to the kinase domains of many plant PRRs; therefore, Pto might act as a 'decoy' for AvrPto virulence targets to activate host immunity (Xing et al., 2007).

The second functional domain of AvrPto, the phosphorylated CTD, is less well understood. Three serine residues at positions 147, 149, and 153 have been identified as the phosphorylation sites *in vitro* (Anderson et al., 2006). One of these sites, S149,

was confirmed to be the *in vivo* phosphorylation site by mass spectrometry. Alanine substitutions at S147 and S149 reduce AvrPto virulence and affect Pto-mediated recognition (Anderson et al., 2006). The molecular and biochemical function of this domain remains elusive although we showed that it does not suppress MAPK activation by certain PAMPs, which is distinct from the function of the CD loop (Yeam et al., 2010). The host kinase activity phosphorylating the AvrPto CTD, termed Avk for AvrPto kinase, is conserved in many plant species but was not observed in yeast (Anderson et al., 2006). Interestingly, the phosphorylation status of this domain is monitored by a novel recognition mechanism found only in certain *Nicotiana* species, termed Rpa for Recognition of phosphorylated AvrPto (Shan et al., 2000; Yeam et al., 2010). Little is known about three aspects of CTD activity: its host targets, and the identities of Avk and Rpa; however, we hypothesize that they might be mechanistically connected. The molecular characterization of these three components will enhance our understanding of the novel host process being targeted by this effector as well as the host immune response countering against bacterial infection.

The focus of this study was to characterize Avk activity in response to PAMPs and pathogenic bacteria, and to identify Avk(s) in hope of elucidating the molecular mechanism by which the phosphorylated AvrPto promotes *Pst* virulence. We screened Arabidopsis protein microarrays for candidate kinases and verified their abilities to phosphorylate the CTD using *in vitro* kinase assays. Calcium-dependent protein kinases (CDPKs) were confirmed as *in vitro* Avks. We further showed the requirement of calcium in phosphorylation of AvrPto. Together, our results indicate that there may be multiple host kinases involved in phosphorylating the AvrPto CTD and that CDPKs may play a significant role in this activity.

Results

PAMPs activate Avk activity

We speculated that since the expression of many kinase genes is upregulated upon exposure of plant cells to PAMPs, AvrPto might take advantage of this elevated kinase activity to phosphorylate its CTD (Navarro et al., 2004; Zipfel et al., 2004; Cohn and Martin, 2005; Yeam et al., 2010). We examined this possibility by monitoring Avk activity from *N. benthamiana* leaf tissue treated with a T3SS mutant strain of *P. syringae* pv. *tabaci* (*Pstab*), *ΔhrcV*. Avk activity was monitored by using an *in vitro* phosphorylation assay with wild-type AvrPto (AvtPto-WT) or the CTD mutant with three Ser residues 147, 149, and 153 substituted to Ala (AvrPto-3XA) as possible substrates. Avk activity was induced significantly as early as 4 hours after inoculation with the non-pathogenic *Pseudomonas* compared to the buffer only ($MgCl_2$) treatment control (Figure 4.1A). This induced phosphorylation was observed only with AvtPto-WT and not with the CTD mutant, AvrPto-3XA (Figure 4.1B). Consistent with our previous results, phosphorylation was significantly diminished in the CTD mutant; however, there is no difference in phosphorylation levels observed between the *Pstab ΔhrcV* treatment and the buffer control treatment when using this substrate (Anderson et al., 2006). Similar results were seen when extracts from *N. benthamiana* plants inoculated with another non-host pathogen *P. fluorescens* was used in the *in vitro* kinase assay (Figure 4.2). Both the T3SS mutant *Pstab ΔhrcV* and *P. fluorescens* were used as the sources of PAMPs. These results indicate that Avk activity is induced by PAMPs.

To further investigate Avk activity upon pathogen inoculation, *N. benthamiana* tissues treated with the pathogenic *Pstab* delivering AvrPto or an empty vector control (EV) were tested in *in vitro* Avk assays with AvrPto-WT or AvrPto-3XA as

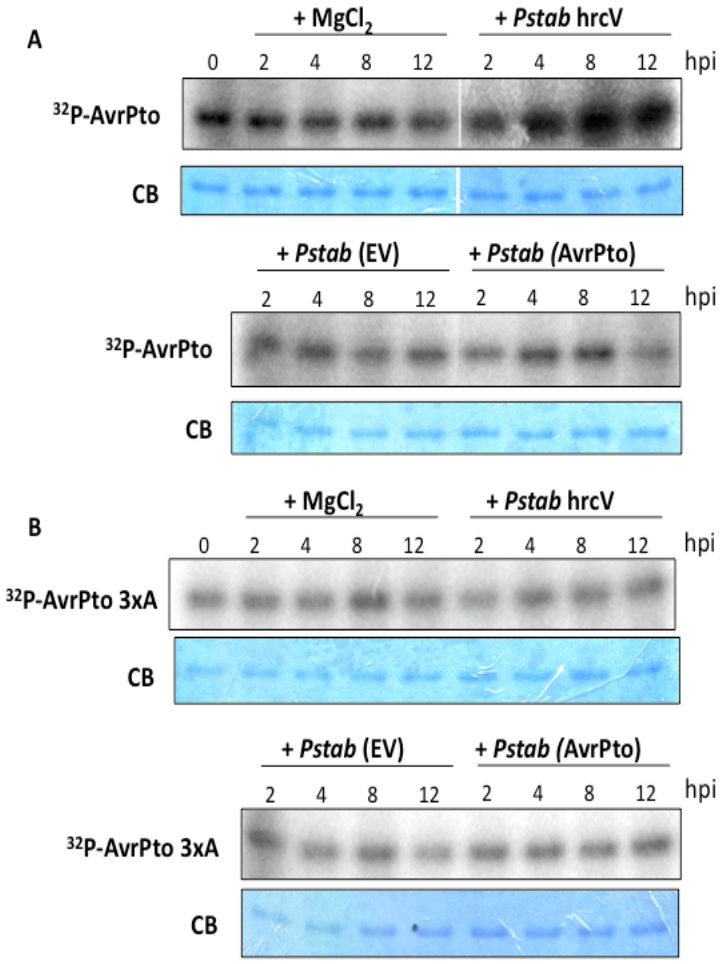

Figure 4.1. Phosphorylation of AvrPto CTD is enhanced by *P. syringae* pv. *tabaci* PAMPs and may be suppressed by type III effectors. Protein extracts were prepared for the AvrPto phosphorylation assay from *N. benthamiana* leaves syringe-infiltrated with 10 mM $MgCl_2$, *P. s.* pv. *tabaci* hrcV (a T3SS mutant and a source of PAMPs), *P. s.* pv. *tabaci* (pDSK519 empty vector), or *P. s.* pv. *tabaci* (pDSK519:AvrPto), 0, 2, 4, 8, and 12 hours after the treatment. AvrPto-WT-FLAG (**A**) or AvrPto-3xA (S147A/S149A/S153A)-FLAG (**B**) was incubated with the protein extracts and [γ-32P]ATP, and then separated by SDS-PAGE. The top panel is an autoradiograph, and the bottom panel is Coomassie blue (CB) staining of the same gel. All bacterial treatments and kinase assays were done at the same time and the experiment was repeated at least twice with similar results.

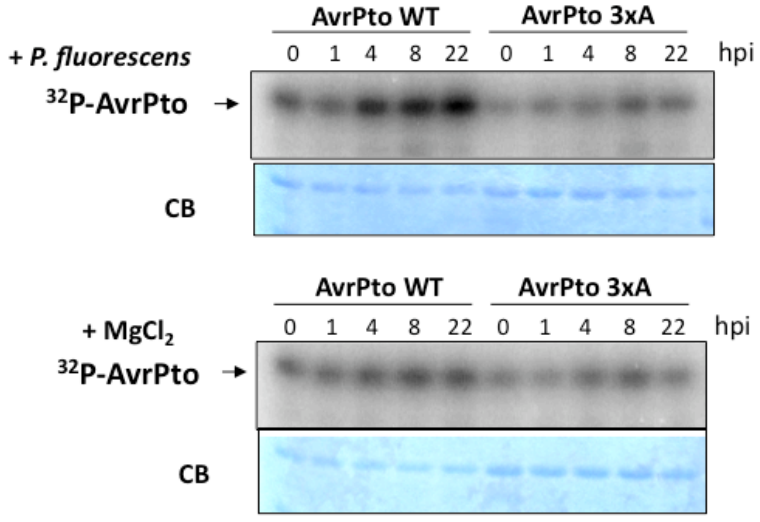

Figure 4.2. Phosphorylation of AvrPto CTD is enhanced by *P. fluorescens* PAMPs. Protein extracts were prepared for phosphorylation assay from *N. benthamiana* leaves syringe-infiltrated with *P. fluorescens* (10^9 cfu/mL) or 10 mM $MgCl_2$, 0, 1, 4, 8, and 22 hours after the treatment. AvrPto-WT-FLAG or AvrPto-3xA (S147A/S149A/S153A)-FLAG was co-incubated with protein extracts and [γ-^{32}P]ATP, and separated by SDS-PAGE gel. The top panel is an autoradiograph, and the bottom panel is Coomassie blue (CB) staining of the same gel. The experiment was repeated at least twice with similar results.

substrates. The pathogenic bacteria, with or without AvrPto, do not affect Avk activity at any time point (Figure 4.1A and B). Based on these results, it appears possible that an unknown effector, or possibly multiple effectors, effectively suppresses the enhancement of Avk activity by PAMPs, which is observed using plant tissue treated with the T3SS mutant of *Pstab* (*ΔhrcV*) or the non-host bacteria *P. fluorescens*. We observed that this effector-mediated suppression of PAMP-induced Avk activity was not enhanced in the presence of AvrPto (Figure 4.1A and 1B).

Phosphorylation of AvrPto also occurs on Ser/Thr residues outside of the CTD *in vivo*

Our previous study reported that AvrPto undergoes multiple modifications in plant cells including N-terminal myristoylation and phosphorylation (Anderson et al., 2006). The 2D gel pattern of the myristoylation mutant, AvrPto-G2A with the second Gly residue mutated to Ala, is similar to that of AvrPto-WT. However, the whole 2D pattern of AvrPto-G2A is shifted to a higher pI and smaller size, which could be due to myristoylation or an unknown myristoylation-independent modification but is not likely to be due to phosphorylation (Anderson et al., 2006). We further examined the phosphorylation status of AvrPto variants *in vivo* including the CD loop mutant (I96A), a CTD deletion (Δ30) that has the last 30 amino acids (135-164) removed from the C terminus, and the myristoylation mutant (G2A). HA-tagged AvrPto and variants were expressed in tomato Rio Grande prf3 (*prf/prf, Pto/Pto*) protoplasts and ^{32}P-orthophosphate was added to detect phosphorylation. AvrPto proteins were immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE and autoradiography. Consistent with our previous results, AvrPto-WT was phosphorylated *in vivo* (Figure 4.3) (Anderson et al., 2006). Although the phosphorylation level of AvrPto-G2A was weaker than AvrPto-WT, the loading of

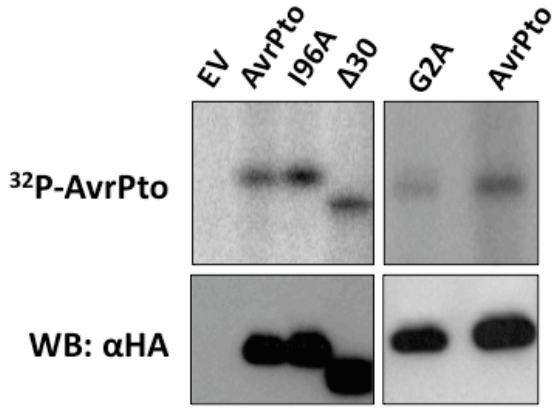

Figure 4.3. AvrPto is phosphorylated outside of the CTD *in vivo.* Tomato Rio Grande-prf3 (*prf/prf, Pto/Pto*) protoplasts were transfected with expression vectors expressing the double Hemagglutinin (dHA)-tagged AvrPto and its variants, G2A, I96A, and Δ30 expressed by the CaMV35S promoter or an empty vector (EV) control. The transfected protoplasts were incubated with ^{32}P-orthophosphate for 8 hours and AvrPto-HA or its variants was immunoprecipitated with anti-HA antibody from the cell extract. SDS-PAGE and autoradiography were used to visualize phosphorylation signals. Upper panels represent the ^{32}P signals and the lower panels show a Western blot using anti-HA antibody.

this variant was also significantly lower. Other AvrPto variants, I96A and Δ30 were phosphorylated to a similar degree to that of AvrPto-WT (Figure 4.3). These results indicated that AvrPto is phosphorylated *in vivo* at Ser/Thr residues outside of the CTD and its overall phosphorylation does not appear to be affected by its membrane localization or the CD loop.

Identification of calcium-dependent protein kinases as potential Avks using a protein microarray

Protein microarrays allow high throughput screening and characterization of molecular interactions and targets of protein kinases in yeast, mammalian, and plant systems (Kung and Snyder, 2006). An Arabidopsis protein microarray has been constructed with proteins purified using a heterologous plant-based expression system, in which recombinant Arabidopsis proteins are produced in *N. benthamiana* using an affinity purification approach (Popescu et al., 2007). Protein kinases expressed in this system are more active compared to those expressed in yeast. We observed while performing *in vitro* kinase assays using recombinant protein kinases that the kinase auto-phosphorylation level was often diminished when a suitable substrate was present (H. P. Nguyen and G. B. Martin, unpublished observation). Based on this observation, we hypothesized that Avk autophosphorylation might be reduced in the presence of its substrate, AvrPto-WT. We performed autophosphorylation assays with or without AvrPto on protein microarray chips that contain 1,133 unique Arabidopsis proteins printed in duplicate, consisting mostly of kinases and transcription factors (Figure 4.4A) (Popescu et al., 2007). The slides were washed extensively after the kinase reaction and the phosphorylation signal intensities were detected by exposure to X-ray

Table 4.1: List of candidate kinases that were tested individually in *in vitro* kinase assays and their characteristics.

AT#	Category*	Protein ID
AT1G34300	decreased	Lectin protein kinase
AT1G74740	decreased	Calcium-dependent protein kinase 30 (CPK30)
AT1G20930	decreased	Cyclin-dependent kinase B2
AT3G45240	decreased	Geminivirus Rep interacting kinase (GRIK)
AT2G17290	decreased	Calcium-dependent protein kinase 6 (CPK6)
AT4G08170	decreased	Inositol 1,3,4-trisphosphate 5/6-kinase
AT1G14370	increased	Protein kinase 2A
AT2G31880	increased	Putative LRR transmembrane protein kinase expressed in response to *P. syringae*
AT5G65530	increased	Putative protein kinase
AT3G08720	increased	Ribosomal-protein S6 kinase
AT5G19150	increased	Carbohydrate kinase
AT5G63710	interact	Putative LRR transmembrane protein kinase
AT3G45780	interact	Blue-light photoreceptor contains a Ser/Thr kinase domain and LOV1 and LOV2 repeats.
AT2G44680	interact	Casein kinase II
AT4G23220	interact	Cysteine-rich receptor-like protein kinase 14 (CRK14)
AT4G23140	interact	Cysteine-rich receptor-like protein kinase 6 (CRK6)
AT5G64960	interact	Cyclin-dependent kinase C2 (CDC2)
AT2G43980	interact	Inositol 1,3,4-trisphosphate 5/6-kinase 4
AT4G08470	interact	MAPKKK10 (At putative MAP3K)
AT3G59790	interact	MAPK10 (AtMAPK-like)

Note:
(*) Category: ‘decreased’ - kinases that have reduced autophosphorylation on the protein microarray in the presence of AvrPto; ‘increased’ - kinases that have higher autophophorylation signal in the presence of AvrPto; ‘interact’ – protein kinases that interact with AvrPto-WT on the protein chip in a binding assay.

Other proteins that interact with AvrPto on the protein microarrays include: AT2G41090 (calmodulin-like protein), AT3G43810 (calmodulin 7), AT4G20780 (calcium-binding protein), AT1G78490 (cytochrome P450 family protein), AT2G35930 (U-box domain containing protein), AT4G34590 (GRAS transcription factor-TF), At1G55580 (bZIP TF), AT4G34590 (bZIP TF), and AT4G35900 (MYB TF).

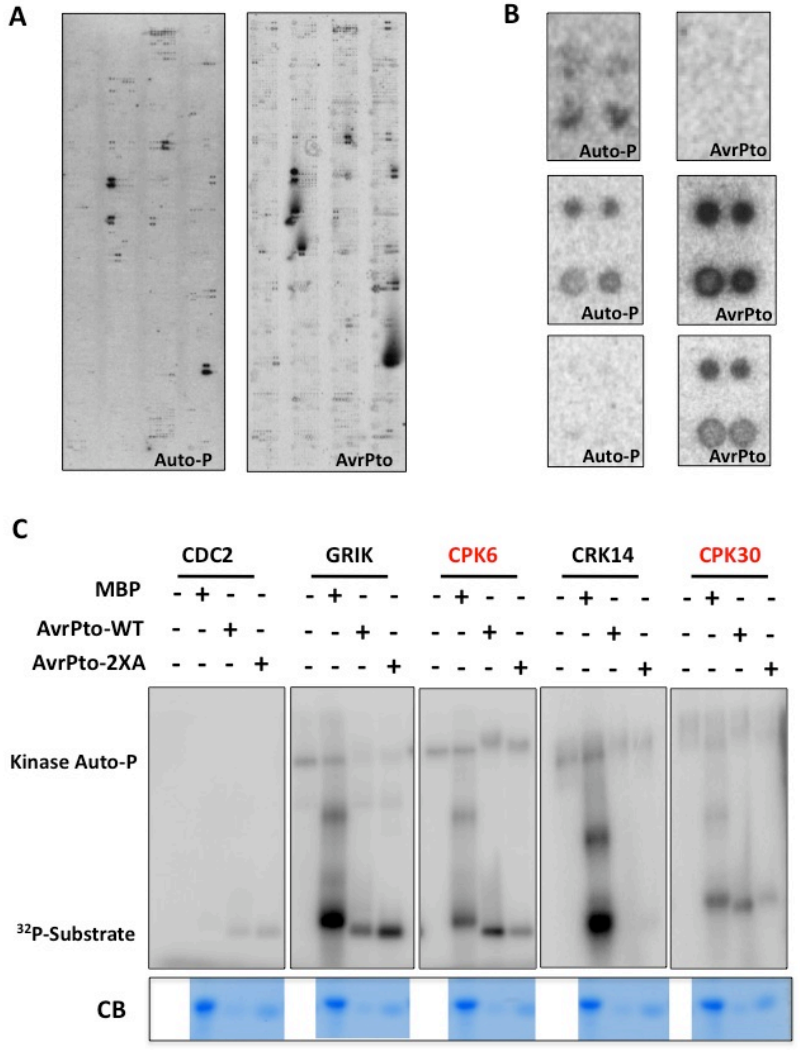

Figure 4.4. Identification of calcium-dependent protein kinases as potential Avks using a protein microarray. (A) Identification of candidate Avk(s) using a protein microarray**.** Protein microarrays containing 1,133 unique Arabidopsis proteins were either incubated with kinase buffer containing [γ-^{33}P]ATP to detect autophosphorylation (Auto-P) or with kinase buffer containing purified AvrPto-WT-FLAG and [γ-^{33}P]ATP (AvrPto) to examine the effect of AvrPto on the kinase autophosphorylation levels. The slides were washed extensively and exposed to X-ray film. Phosphorylation signals were analyzed by GenePix. **(B)** Examples of three different alterations in kinase autophosphorylation levels observed when AvrPto was present. The top panel represents proteins that have reduced phosphorylation in the presence of AvrPto. The middle panel shows example of proteins that have enhanced autophosphorylation level in the presence of AvrPto. The bottom panel shows kinases that only show detectable autophosphorylation in the presence of AvrPto. **(C)** Examples of *in vitro* kinase assays with candidate kinases. Arabidopsis AtCPK6 and AtCPK30 (in red) were identified as potential Avks. Individual kinases were

expressed in *Nicotiana benthamiana* and purified using the TAP protein expression and purification system. Purified kinases were subjected to *in vitro* kinase assays with myelin basic protein (MBP), AvrPto-WT-FLAG, and AvrPto-2XA-FLAG as substrates. Upper panel is an autoradiograph and the bottom panel is Coomassie blue (CB) staining of the substrates.

film and analyzed using GenePix. One hundred and eight protein kinases showed detectable phosphorylation signals on at least one of the two assay conditions (with or without AvrPto). Among them, there were 6 kinases that exhibit a reduction in autophosphorylation when AvrPto was present in the kinase buffer (Figure 4.4B). Interestingly, aside from the many kinases that did not show any significant difference between the two assay conditions, there were 35 kinases that showed enhanced autophosphorylation level in the presence of AvrPto. Among these, autophosphorylation signals of 27 kinases were only detected when AvrPto was in the reaction buffer. Since it was reported previously that substrates could enhance autophosphorylation and activation of certain kinases, we decided to include a few of these 35 kinases together with the 6 that showed reduced autophosphorylation levels in our further analysis to identify Avk (Table 4.1) (Jakobi et al., 2000).

The binding affinity of a kinase to its substrate varies in different kinase-substrate pairs. In some cases, the interaction is only transient (Manning and Cantley, 2002). However, since Avk activity was pulled down successfully with AvrPto full length but not with the C-terminal deletion protein (I. Yeam and G. B. Martin, unpublished result), we hypothesize that AvrPto might physically interact with its kinase(s). Using a protein microarray with the same set of proteins as described above, we screened for proteins that bind to AvrPto. We identified 9 kinases among 18 proteins that interact with AvrPto on the protein chips (Table 4.1). The other proteins include transcription factors, a calcium-binding protein, a U-box containing protein, and others. The kinases identified in this experiment were also included in the Avk candidates list (Table 4.1).

Our previous studies indicate that phosphorylation at two Ser residues, 147 and 149, is sufficient for the CTD virulence in tomato and its recognition by tobacco Rpa (Nguyen et al., 2010; Yeam et al., 2010). We therefore used AvrPto with Ala substitutions at S147 and S149 (AvrPto-2XA) in subsequent assays instead of AvrPto-3XA. AvrPto-2XA is phosphorylated similarly to AvrPto-3XA in *in vitro* kinase assays, which is significantly less than AvrPto-WT (data not shown). Kinases of particular interest were those that phosphorylated AvrPto-WT more strongly than AvrPto-2XA. Candidate recombinant protein kinases were expressed and purified according to a published protocol (Popescu et al., 2007). Individual kinases were then subjected to *in vitro* kinase assays with AvrPto-WT, AvrPto-2XA, or myelin basic protein (MBP) as the substrate, or kinase buffer only to detect autophosphorylation. As shown in the examples in Figure 4.4 C, kinases tested either did not phosphorylate AvrPto while autophosphorylating or phosphorylating MBP or they phosphorylated both AvrPto-WT and 2XA similarly. Only calcium-dependent protein kinases (CDPK), AtCPK6 and AtCPK30, showed the expected Avk phenotype, which was weak phosphorylation of AvrPto-2XA as compared to AvrPto-WT.

Multiple AtCPKs from clade 1 and 3 phosphorylate the AvrPto CTD

The AtCPK gene family consists of 34 members that are classified based on a phylogenetic analysis into 4 different clades (Figure 4.5A) (Cheng et al., 2002). To test the specificity of phosphorylation of the AvrPto CTD, we examined CDPKs derived from the different clades for their ability to phosphorylate the CTD. Only

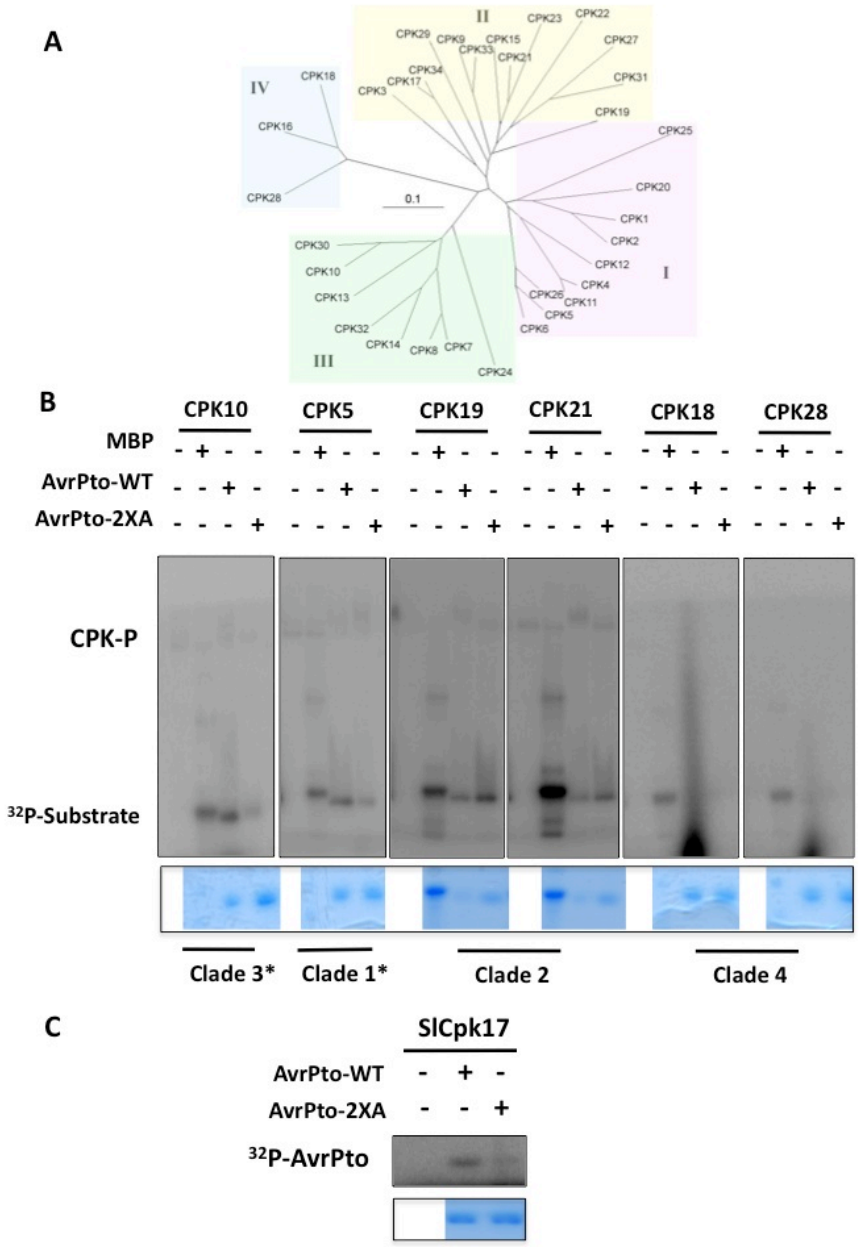

Figure 4.5: AtCPKs f rom clade 1 and 3 phosphorylate the CTD *in vitro*. (A) Phylogenetic analysis of Arabidopsis CDPK protein family. Adapted from Cheng et al. 2002. **(B)** Arabidopsis CDPKs from clade 1 and 3 phosphorylate AvrPto's CTD while CPKs from clade 2 and 4 do not. The upper panel is an autoradiograph and the bottom panel is Coomassie blue staining of the substrates (MBP, AvrPto-WT-FLAG, or AvrPto-2XA-FLAG). **(C)** Tomato SlCpk17, homolog of AtCPK30, also phosphorylates the AvrPto CTD *in vitro*.

CDPKs from clade 1 and 3 specifically phosphorylated the CTD (Figure 4.5B and Table 4.2). CDPKs from clade 2 phosphorylated AvrPto-2XA to a similar degree as AvrPto-WT while CDPKs from clade 4 did not phosphorylate either substrate (Figure 4.5B).

Since Avk activity is observed in many plant species, the kinases that phosphorylate the AvrPto CTD are probably conserved in various plants. We examined whether a previously characterized CDPK from tomato could also phosphorylate the AvrPto CTD. Full-length *SlCpk17*, a tomato homolog of *AtCPK10* and *AtCPK30* (clade 3), was cloned into the expression vector pYL436 to produce recombinant protein using the same approach that was used for the Arabidopsis kinases described above. SlCpk17 indeed phosphorylated AvrPto at the CTD (Figure 4.5C). A recent genome-wide annotation of tomato kinases shows that tomato has 44 CDPK genes (Zhangjun Fei, unpublished data). SlCpk17 together with 6 other SlCpks are grouped together with clade 3 AtCPKs in this phylogenetic analysis. Although we have not tested whether other SlCpks could also phosphorylate the AvrPto CTD, it is reasonable to expect that additional tomato SlCpks belonging to clade 1 and 3 will also be able to phosphorylate AvrPto in the same manner as the Arabidopsis homologs.

In vitro phosphorylation of the AvrPto CTD is Ca^{2+}-dependent

CDPKs have been proposed to function in many signal transduction pathways that respond to elevated cytosolic Ca^{2+} levels (Harmon et al., 2000). Their activation usually requires the presence of Ca^{2+}. We examined if phosphorylation of the AvrPto CTD is Ca^{2+}-dependent in the *in vitro* assay using recombinant proteins. Indeed,

phosphorylation of AvrPto-WT by purified CPK5 and CPK32 was completely abolished when EGTA, a calcium chelator, was added to the reaction buffer but was not affected by the presence of Ca^{2+} in the form of $CaCl_2$ (Figure 4.6A).

Table 4.2: List of AtCPKs tested for phosphorylation of the AvrPto CTD and their characteristics.

CPK#	Clade	Phosphorylates CTD?	Expressed in mesophyll cells? *	Upregulated by flg22? **
CPK2	1	ND	yes	yes
CPK20	1	yes	no	NA
CPK6	1	yes	yes	yes
CPK4	1	yes	yes	NA
CPK5	1	yes	yes	yes
CPK19	2	no	no	NA
CPK21	2	no	yes	NA
CPK22	2	no	yes	NA
CPK13	3	yes	yes	NA
CPK10(1)	3	yes	yes	yes
CPK30	3	yes	yes	NA
CPK32	3	yes	yes	yes
CPK18	4	no	no	NA
CPK28	4	no	yes	yes

Note: *Expression of CPKs in mesophyll protoplasts based on qRT-PCR (Boudsocq et al., 2010). **Genes induced in response to flg22 treatment using an Arabidopsis full genome Gene Chip ATH1 (Affimetrix) (Navarro et al., 2004 and Zipfel et al., 2004). NA: information not available.

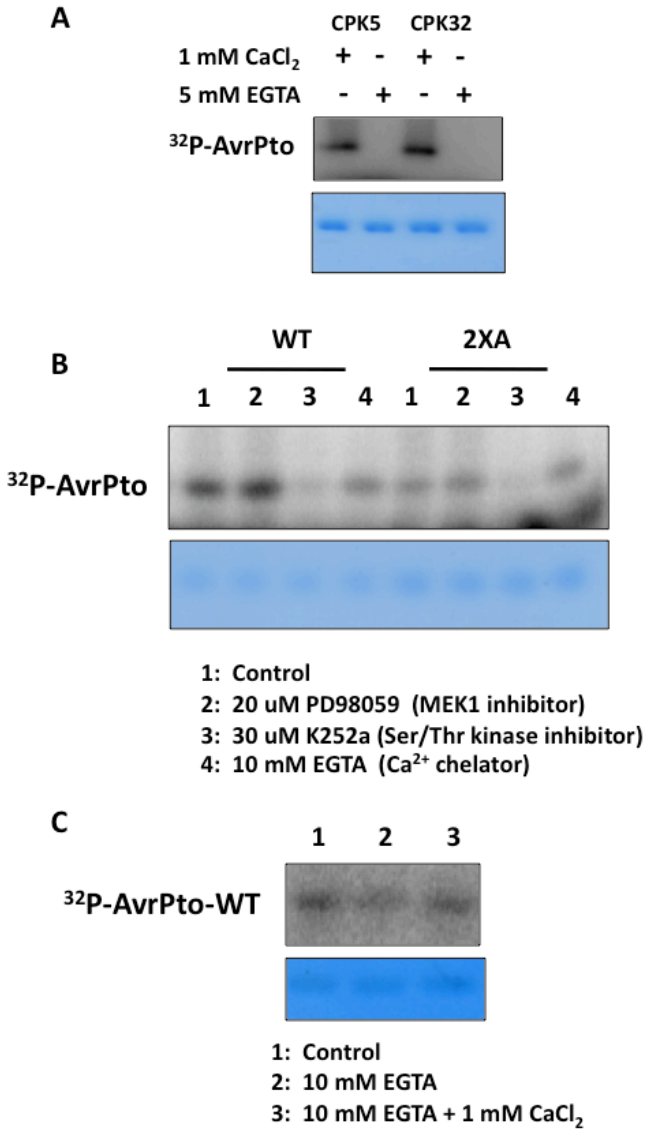

Figure 4.6: *In vitro* phosphorylation of the AvrPto CTD is Ca^{2+}-dependent. (A) AtCPK5 and AtCPK32 do not phosphorylate AvrPto in the absence of Ca^{2+} (+ 5 mM EGTA). **(B)** Avk activity from *N. benthamiana* extract is diminished in the presence of EGTA, a calcium chelator, and K252a, a Ser/Thr kinase inhibitor but not in the presence of PD98059, a selective MAPK pathway inhibitor. **(C)** Avk activity from *N. benthamiana* extract is Ca^{2+}-dependent. $CaCl_2$, used here as a Ca^{2+} source to sequester EGTA, is added to the kinase buffer to show that Ca^{2+} is the ion needed for Avk activity from plant extract *in vitro*.

Next, we investigated whether Avk activity from plant extracts was dependent on Ca^{2+}. Extracts of *N. benthamiana* were used in an *in vitro* phosphorylation assay with AvrPto-WT or AvrPto-2XA as the substrate. Avk activity was noticeably reduced in the presence of EGTA and almost abolished in the presence of K252a, a Ser/Thr kinase inhibitor (Figure 4.6B). PD98059, a selective inhibitor of the mitogen-activated kinase (MAPK) pathway, did not affect Avk activity. EGTA did not alter the phosphorylation level of AvrPto-2XA, indicating that phosphorylation of only the CTD is Ca^{2+}-dependent. To confirm that the calcium ion (Ca^{2+}), and not other ions such as magnesium ion (Mg^{2+}), is required for Avk activity in plant extracts, we added $CaCl_2$ together with EGTA to the kinase buffer. While EGTA reduced the phosphorylation of AvrPto-WT, the addition of $CaCl_2$ restores Avk activity to the level of the water control (Figure 4.6C). These results indicate that Avk activity from plant extract, as examined using *in vitro* kinase assays, is indeed Ca^{2+}-dependent.

Discussion

AvrPto has been studied extensively since the gene was cloned almost two decades ago, and yet there are still many unknowns regarding its molecular and biochemical functions in the host cell. Besides the involvement of the CD loop in disrupting PRR complexes, which has been well characterized, the function of the CTD remains largely unknown. This domain appears to utilize a host kinase activity, Avk, to facilitate its virulence activity (Anderson et al., 2006). Certain *Nicotiana* species have evolved to recognize the phosphorylated CTD (Yeam et al., 2010). We have shown here that Avk activity in plant extracts is enhanced by PAMP treatments suggesting that Avk might be involved in PTI-mediated perception and/or signaling pathways. Treatments with pathogenic bacterial strains suppressed the induction of Avk activity by PAMPs indicating a potential role of effectors in this suppression activity. Using

protein microarrays, we generated a list of candidate kinases that either interact with AvrPto or have altered autophosphorylation level in the presence of AvrPto. By screening these candidates, we identified CDPKs as potential Avks. Subsequently, we provided evidence that *in vitro* phosphorylation of the CTD by plant extracts is indeed calcium-dependent, further supporting a role of CDPKs in phosphorylating the AvrPto CTD.

Avk activity is present in many plant species without any pre-treatment, however, this activity can be enhanced by PAMPs (*Pstab ΔhrcV* and *P. fluorescens*) (Figure 4.1 and 4.2). Significantly increased kinase activity was detected after PTI-induction, which could be due either to enhancement of enzyme activity or to increased transcription and protein expression/accumulation after the treatment. Our observation that at least a 3-4 hour period is needed to detect the increased kinase activity probably supports the latter scenario; however, further experiments are needed to test this hypothesis. Previous studies have shown that many kinase genes are transcriptionally induced upon PAMP treatment (Navarro et al., 2004; Cohn and Martin, 2005). Many of these kinases, including many classes of RLKs and lectin-kinases, are suggested to have roles in plant defense. The fact that T3Es may play a role in interfering with enhancement of Avk activity by PAMP treatment further supports involvement of Avk in PTI. The presence of AvrPto (in *Pstab*-AvrPto) does not further reduce Avk activity as compared to *Pstab*-EV. This may be because maximal suppression is already achieved by other effectors, although it is also possible that AvrPto plays no role in suppression of the PTI pathways that are involved in Avk induction.

One of major activities of T3E's, including the AvrPto CD loop, is to suppress PTI, which typically leads to the suppression of many host kinases (He et al., 2006; Cunnac et al., 2009). This activity would appear to conflict with the fact that AvrPto

requires host kinase activity to phosphorylate its CTD. It has been reported that the host responses to PAMPs could be detected as early as minutes after treatments of cell cultures or seedlings with elicitors (Navarro et al. 2004; Zipfel et al. 2004). Bacterial effectors have to be synthesized *de novo* and delivered into the host cell though the T3SS, which is constructed upon the induction by cues present in the plant apolastic environment. It has been estimated that about two hours is needed for the complete formation for the molecular syringae upon the based on analysis of the construction of the T3SS (Pozidis et al., 2003). Therefore, it is likely that, upon being delivered into the plant cell, AvrPto is immediately phosphorylated by the abundant kinase activity that has been induced by PAMPs. AvrPto phosphorylation could occur before T3SS effectors starts targeting the host PTI pathways, which leads to suppressing the activation of PTI-related kinases. Consistent with our previous study, we observed that AvrPto myristoylation mutant (G2A) is also phosphorylated *in vivo* (Anderson et al., 2006). This result supports the possibility that AvrPto is phosphorylated on the CTD and then targeted to the plasma membrane where the CD loop and the CTD function to promote bacterial virulence.

It was unexpected that while significant reduction in phosphorylation is consistently observed when important Ser residues at the CTD are substituted for Ala in *in vitro* kinase assays using plant extract, we could not detect any significant reduction in phosphorylation of the CTD truncation in the *in vivo* kinase assay as compared to the WT (Figure 4.3). AvrPto has 19 Ser and Thr residues outside of the CTD that could potentially be phosphorylated. Our *in vivo* data suggests that these other sites are phosphorylated strongly *in vivo* and that even truncating the CTD does not have a significant effect on the overall phosphorylation level of AvrPto. In fact, we observed significant CTD-independent phosphorylation of AvrPto by multiple kinases (Figures 4.4 and 4.5). These kinases could be responsible for phosphorylating the non-

CTD Ser/Thr residues *in vivo* (Figure 4.3). However, although AvrPto might be phosphorylated at other sites than S147 and S149, the phosphorylation of only these two serines by Avk has been demonstrated experimentally to have significant biological relevance to the effector's activities *in planta* (Anderson et al., 2006; Yeam et al., 2010). The discrepancy between our *in vivo* and *in vitro* data could be due to certain conformational changes that allow the CTD to be more exposed, as compared to the other possible phosphorylation sites, for kinase accessibility in our *in vitro* kinase assay condition. Whatever the underlying reason, the ability to robustly detect CTD phosphorylation *in vitro* offers many advantages for the characterization of Avk.

Protein microarrays have been used extensively in the characterization of proteomic networks including the interactions between protein-protein, protein-nucleic acids, and protein-phospholipids (Kung and Snyder, 2006; Popescu et al., 2007). They are also useful in screens for substrates of individual kinases (Ptacek et al., 2005). In this study, we utilized the protein chips to identify potential kinase(s) for AvrPto. The kinases that were positive for the 'Avk phenotype', AtCPK6 and AtCPK30, are members of the group of kinases that showed a reduction in autophosphorylation level (on the protein chip) when AvrPto is present. Indeed, we also observed a reduction in GRIK autophosphorylation levels when AvrPto is the substrate in an *in vitro* kinase assay (Figure 4.4). In the case of CDPKs, we observe a mobility shift when AvrPto is present. However, whether or not this mobility shift in CDPK has any biological relevance remains to be tested.

Although some kinases that bind to AvrPto on the protein chip, such as CDC2 and CRK14, could phosphorylate AvrPto, their phosphorylation was not specifically on the CTD (Figure 4.4C). Many of the interacting kinases are receptor-like kinases (RLKs) (Table 4.1). Their interactions might therefore be mediated through binding to the CD loop (Xing et al., 2007). It would be interesting to test if these RLKs are

involved in PTI and if they are indeed targeted by the CD loop. Overall, our results show that protein microarrays can be used effectively to identify candidate kinases for type III effectors.

CDPKs are protein kinases that are widely distributed in the plant kingdom (Cheng et al., 2002). Other than plants, CDPKs have been identified in some protozoans but they are not present in other eukaryotic organisms such as yeast, flies, nematodes, and human (Cheng et al., 2002). In Arabidopsis, the CDPK gene family includes 34 members. CDPKs typically contain, in order, an N-terminal variable domain, a Ser/Thr kinase domain, an autoinhibitory domain, and a C-terminal intrinsic Ca^{2+}-activation domain with four EF hand Ca^{2+}-binding sites (Harper et al., 1991). CDPKs have been suggested to act as key regulators in many plant responses including hormone signaling, growth and development, guard cells and stomata movement, and biotic and abiotic stresses (Cheng et al., 2002; Mori et al., 2006).

The roles of CDPKs in plant defense have been demonstrated in a few studies in both Arabidopsis and some Solanaceous plants (Romeis et al., 2000; Kobayashi et al., 2007). Transcriptional studies in both Arabidopsis and tomato have shown that some CDPK genes are upregulated in response to PAMPs (Navarro et al., 2004; Zipfel et al., 2004). In a recent study, members of Arabidopsis CDPK family, AtCPK4, 5, 6, and 11 were shown to constitute a signaling pathway downstream of PAMP-receptors and independent of the well studied MAPK signaling pathway (Boudsocq et al., 2010). In addition, many CDPKs contain an N-terminal myristoylation motif and an additional palmitoylation motif, which suggests plasma membrane localization (Cheng et al., 2002). As mentioned earlier, CDPKs are not found in yeast, and our previous study has shown that yeast does not phosphorylate AvrPto (Anderson et al., 2006). With all of the characteristics mentioned, CDPKs appear to be very good candidates for Avk.

Although CDPKs have been implicated to have roles in a variety of plant processes *in vitro*, there are only a few examples where a CDPK has been experimentally demonstrated *in vivo* to be directly involved in regulating a biological process (Romeis et al., 2001; Mori et al., 2006; Kobayashi et al., 2007; Boudsocq et al., 2010). The lack of dominant negative forms and specific inhibitors, and functional redundancy are the major challenges in studying the functions of CDPKs *in planta* (Cheng et al., 2002). We examined whether extracts from existing CPK mutant lines of Arabidopsis, *cpk5, 6,* or *11,* and the triple mutant *cpk5/6/11*, showed reduced AvrPto phosphorylation as compared to wild type plant extracts in an *in vitro* phosphorylation assay (Boudsocq et al., 2010). However, no consistent reduction in AvrPto phosphorylation was observed with any of these lines (data not shown). Since all purified CDPKs that we tested from clade 1 and 3 phosphorylate the AvrPto CTD *in vitro*, we suspect other CDPKs from these clades may have Avk activity. This could be the reason why we could not observe any effect from *cpk* mutant plants on Avk activity in plant extract, which prevents us from examining the effect of lacking CDPKs on the CTD's virulence in Arabidopsis as well as Rpa in *Nicotiana* species. However, we found that *in vitro* Avk activity from plant extracts is Ca^{2+}-dependent. In the presence of the Ca^{2+} chelator, EGTA, phosphorylation of AvrPto-WT is significantly diminished while phosphorylation of AvrPto-2XA is not affected (Figure 4.6B). CTD phosphorylation is restored when Ca^{2+} (in the form of $CaCl_2$) is added into the kinase buffer together with EGTA, which strongly implicates the role of Ca^{2+} in this modification (Figure 4.6C). It is important to note that Ca^{2+} addition is not required to detect Avk activity by plant extracts or by purified CDPKs, which indicates that Ca^{2+} present in the protein preparation is sufficient to activate the kinases. The Ser/Thr kinase inhibitor, K252a, almost completely abolished AvrPto phosphorylation in both the WT and the CTD mutant forms, which further supports

that AvrPto is phosphorylated outside of the CTD by Ser/Thr kinases (Figure 4.6B).

This study shows, for the first time, the identification of plant kinases that phosphorylate the virulence determinant of a bacterial effector. Our results indicate that there are multiple but specific kinases that could phosphorylate the AvrPto CTD. It is interesting that AvrPto has evolved to be a specific substrate of host kinases to facilitate its function. We hypothesize that by being a suitable substrate of a large and conserved family of kinases, AvrPto is more likely to get activated once delivered into the plant cells, especially since the abundance of these kinases already increases during the PTI response (Navarro et al., 2004; Cohn and Martin, 2005).

The host target of AvrPto CTD virulence activity remains unknown. It is possible that the CTD is both phosphorylated by CDPKs and then targets them to promote bacterial virulence. This mechanism would be analogous to some animal pathogen effectors. For example, type IV effector CagA from *H. pylori* is phosphorylated by the host Src kinase family and in turn deactivates Src's kinase activity via a negative feedback loop (Selbach et al., 2002; Selbach et al., 2003). Src kinase inactivation leads to the dephosphorylation of corcactin, which results in host cell actin cytoskeletal rearrangement. The reduction in CPKs' autophosphorylation levels in the presence of AvrPto suggests that AvrPto can act as a competitive inhibitor of CPKs. Alternatively, AvrPto might become activated via CPK-mediated phosphorylation in order to manipulate non-CPK host targets. The enteropathogenic bacteria *Escherichia coli* effector Tir, a translocated intimin receptor, is phosphorylated by a host kinase activity (Kenny, 2002). The phosphorylation of Tir is required for its actin nucleating function, which is needed for bacterial adhesion. These and other scenarios for AvrPto CTD activity will need to be investigated for the mechanism of the CTD virulence activity to be understood.

Materials and Methods

Protoplast transfection and *in vivo* kinase assay

Tomato Rio Grande-prf3 (*prf/prf Pto/Pto*) protoplasts were isolated and transfected according to a published protocol (Nguyen et al., 2010). *In vivo* labeling with ^{32}P-orthophosphate and immunoblotting were performed using a similar protocol as used in Anderson et al., 2006.

In vitro kinase assay using plant extracts

About 100 mg of frozen *N. benthamiana* tissue was ground with copper beads and thawed in 250 uL of ice-cold GTEN extraction buffer (25 mM Tris HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, and 10% glycerol) containing 10 mM DTT, 2% w/v PVPP, and 1X plant general protease inhibitor (Sigma). Cell debris was collected by centrifugation at 13,000g for 5 minute. The kinase reaction contained 1 ug of purified AvrPto-FLAG, five microliter clear plant extract, and ten microliter kinase buffer (25 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 20 uM ATP, and 0.2 uCi ^{32}P-gamma ATP). The reaction mixture was incubated for 10 minutes at room temperature (RT) and stopped by adding 3X SDS-sample buffer and separated by SDS-PAGE. For *in vitro* kinase assays using purified kinases, individual TAP- tagged proteins were produced using a described protocol (Popescu et al., 2007). Purified protein kinases were added to the kinase reaction buffer (10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 mM HEPES pH 7.4, 1 mM DTT, 20 uM ATP, and 0.2 uCi ^{32}P-gamma ATP) with purified substrates (1 ug of AvrPto-FLAG proteins and 0.25 mg/mL of myelin basic protein, MBP). Kinase inhibitors, K252a (Sigma) and PD98059 (Promega), and Ca^{2+}-chelator EGTA were added to the kinase buffer to the proper concentrations. The reaction was stopped after a 30-minute incubation at RT and

separated by SDS-PAGE. ^{32}P-labelings of AvrPto and other proteins were detected by a phosphor imager (GE Healthcare). The same reaction without ^{32}P-gamma ATP was separated on a different gel for Coomassie blue staining to detect protein loading.

Protein microarray: Kinase assay and binding assay

For the kinase assays, 1,133 purified proteins were printed on UltraGAPS slides from Corning (Corning, NY) in duplicate (Popescu et al., 2007). Two hundred microliter of kinase buffer (25 mM Tris. HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 20 uM ATP, and 20 uCi ^{33}P-gamma ATP) with or without 5 ug of purified AvrPto-FLAG was overlayed on each protein slide covered with a HybriSlip and incubated for 1 hour at 30^{o}C in a wet chamber. The slides were washed in three times for 30 minutes each time in wash buffer (50 mM Tris and 0.5% SDS). Excess solution was removed by a three-minute centrifugation at 2,000 rpm in a 50 mL Falcon tube. The dry slides were exposed on X-ray film. The film was scanned with the highest resolution (transparency setting) and the digitized image was analyzed with GenePix (Axon Instruments, Sunnyvale, CA). Final intensity of each spot equals to the actual intensity minuses the background intensity. Intensities of the same protein spot between the two conditions (autophosphorylation-no AvrPto and –with AvrPto) were compared.

For binding assay, protein microarray slide used was similar to those used in previous study (Popescu et al., 2007). The slide was blocked for 1 hour in Pierce Blocking Solution (Thermo Fisher Scientific, Rockfort, IL) and washed once with probing buffer (50 mM Tris. HCl pH 7.5, 5 mM $MgCl_2$, 0.5 mM DTT, and 1 mM EGTA). Two hundred microliter of probing buffer containing 5 ug of purified AvrPto-FLAG or no AvrPto-FLAG control was applied onto each slide and covered with HybriSlip. The slides was then incubated with α-FLAG antibody (Sigma) for 2 hours and washed 3 times (10 minutes each) with TBST. Then, the slides were incubated

with Cy5 labeled secondary antibody for one hour and washed 3X10 minutes, spun dried, and scanned in a GenePix 4200A scanner. Spot intensities were analyzed using methods as described in Popescu et al., 2007.

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CHAPTER 5

PERSPECTIVES AND FUTURE DIRECTIONS

Despite having been studied intensively in the past two decades, AvrPto continues to reveal new information about its virulence functions in plant cells and the host surveillance mechanisms that monitor its activity. In recent years, several studies have shown AvrPto activity in suppressing PTI by disrupting PRR complexes, which is attributable mostly to the CD loop function (Shan et al., 2008; Xiang et al., 2008). In this dissertation research, I focused on characterizing the other virulence determinant of AvrPto, the phosphorylated C-terminal domain (CTD).

As discussed in Chapter 2, the CD loop and the CTD contribute to overall virulence activity of AvrPto in an additive manner; however, the mechanism by which the CTD promotes bacterial virulence is distinct from that of the CD loop. This chapter also describes the characterization of a novel resistance found only in *Nicotiana* species that specifically recognize the CTD, termed Rpa. In Chapter 3, I have shown that the two virulence determinants of AvrPto are functionally conserved in AvrPto homologs from other *P. syringae* pathovars, indicating that they may target similar host processes in their respective host plants. In Chapter 4, I present the characterization of a host kinase activity, Avk, that phosphorylates the C-terminus of AvrPto, and the identification of calcium-dependent protein kinases as candidate Avks.

The unknown virulence target(s) of AvrPto's CTD

The CTD promotes bacterial virulence in susceptible tomato plants independently of the CD loop (Yeam et al., 2010). However, the biochemical function and molecular

target(s) of this domain in the plant cell remain elusive. We know that the CTD needs to be activated by an unknown host kinase activity for its function (Anderson et al., 2006; Yeam et al., 2010). Phosphorylation by host kinase activity is not unique to AvrPto. There are many examples of effectors from both plant and animal bacterial pathogens that get phosphorylated once delivered into the host cells, indicating that this is a common phenomenon among many secreted effectors (Backert and Selbach, 2005). Unlike the functions of many phosphorylated effectors from animal pathogens, which are relatively well characterized, the functions of the plant pathogen counterparts are not as well understood (Backert and Selbach, 2005). Among the plant bacterial effectors that are known to be phosphorylated, which include *P. syringae* effectors AvrPto, AvrPtoB, AvrB, and *Rhizobium* sp. NGR234 effectors NopL and NopP, AvrPto and AvrPtoB are the only ones whose phosphorylation sites have been mapped and shown to contribute directly to the effector virulence and avirulence activity in host cells (Anderson et al., 2006; Xiao et al., 2007). Understanding the functions of these phosphorylated effectors will shed light on a novel type of function deployed by bacterial effectors.

In Chapter 2, I have shown that the CTD does not promote virulence via suppression of PTI induced by certain PAMPs, which is instead due to the CD loop (Yeam et al., 2010). In another study, the CD loop was indicated to be responsible for AvrPto function in disrupting defense-related miRNA pathways (Navarro et al., 2008). Our results do not exclude the possibility that the CTD is involved in suppression of other currently unknown PTI pathways. In order to gain more insight into the CTD virulence activity, I believe it would be useful to perform a comprehensive transcriptional profiling of plant tissues in response to different AvrPto variants. This approach would allow us to distinguish the different host processes affected by the CD loop versus the CTD. In addition, we might be able to identify a specific molecular

marker for the CTD virulence activity from such a transcriptomic study. Having a molecular marker would aid in the characterization of the CTD virulence target(s) once identified.

We hypothesize that the host virulence target(s) of the CTD binds to AvrPto in a phosphorylation-dependent manner. An earlier study from our lab has identified four Api proteins, for AvrPto interacting proteins, from a yeast two-hybrid (Y2H) screen using AvrPto as a bait, including two Rab-related small GTPases, a stress-induced protein, and an N-myristoytransferase (Bogdanove and Martin, 2000). However, these proteins are not likely to be the CTD virulence targets since their interactions with AvrPto are not CTD-dependent. A recent Y2H screen was carried out in our laboratory using a phosphomimetic version of AvrPto, AvrPto-2XD with Asp substitutions at Ser147 and Ser149, as the bait and a *Nicotiana sylvestris* cDNA as a prey library. The screen yielded some interesting host proteins, but much still needs to be done to confirm any one of these as a true host target of the CTD (I. Yeam and G. B. Martin, unpublished data). Another set of AvrPto-interacting proteins has been identified using an Arabidopsis functional protein microarray (J. Anderson, S. Popescu, and G. B. Martin, unpublished data). Many of these interacting proteins have been suggested or shown to be involved in plant defense. Further characterization of these proteins is needed to verify their role in plant defense pathways and their possible involvement in CTD virulence promoting mechanisms.

Interestingly, the phosphorylation status of the CTD is monitored by Rpa which has only been identified in *Nicotiana* species but not in other *Solanaceae* genera (Yeam et al., 2010). Therefore, some of AvrPto interactors from the Y2H screen with *N. sylvestris* cDNA library, which has Rpa phenotype, could be involved in Rpa-mediated recognition of the CTD, either by being the resistance protein itself or by being a component of the resistance protein complex. The eventual identification and

characterization of the CTD virulence target(s) and the host resistance recognizing it will elucidate key molecular mechanisms of the co-evolutionary 'arms race' between the host and the pathogen.

Although I showed that the CD loop and the CTD appear to target different host pathways to promote virulence, there is some overlap between the known virulence functions of the two domains (Chapter 2). It was shown that AvrPto induces expression of genes involved in ethylene production and thereby ethylene production (Cohn and Martin, 2005). Ethylene production contributes to AvrPto (and AvrPtoB) virulence functions for the enhancement of bacterial growth and disease symptoms. Therefore, it was proposed that AvrPto may exploit the host ethylene production pathway to promote disease symptoms (Cohn and Martin, 2005; Lin and Martin, 2005). In Chapter 2, I examined whether it is the CD loop or the CTD that is responsible for this activity and found that both virulence determinants contribute additively to the host ethylene production. The host process that AvrPto directly targets to induce ethylene production remains unknown; however, it appears that this activity is at a convergent point of both domains' virulence functions and the full effect could only be achieved when both domains are present.

There are many scenarios of how the two AvrPto virulence domains might manipulate the host hormone synthesis pathway to its advantage. It is possible that a downstream effect of the domains virulence targets leads to the induction of ethylene production, which could include the induction of a transcription factor that activates the ACC oxidase (*ACO*) gene. Alternatively, the two domains virulence activities could account for the promotion of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) phosphorylation (Liu and Zhang, 2004). It is important to note that another *P. syringae* pv. *tomato* effector, AvrPtoB, also induces ethylene production similarly to AvrPto (Cohn and Martin, 2005). This effector has been shown to target many of the

same host processes as the AvrPto CD loop such as targeting the host PRRs and suppression of PTI (He et al., 2006; Shan et al., 2008). Therefore, it seems likely that enhanced ethylene production is a convergent downstream event of these two effectors virulence activity. Alternatively, both domains of AvrPto and AvrPtoB may directly control certain aspects of ethylene biosynthesis. Interestingly, a key component of the ethylene biosynthesis pathway has been identified to interact with AvrPto from the Y2H screen mentioned above (I. Yeam and G. B. Martin, unpublished data). It will be interesting to test if AvrPto targets this protein *in planta*, and if this binding activity results in altered ethylene production.

Identification of Avk(s)

Examples from animal pathogens reveal that phosphorylated effectors could either directly manipulate the host kinase(s) phosphorylating it or target other host processes that are not related to the kinases themselves (Backert and Selbach, 2005). It appears that the phosphorylated effectors recruit a variety of host proteins, some of which have been shown to be the virulence targets of these effectors *in vivo* (Kenny, 2002). The phosphorylated CTD of AvrPto might promote bacterial virulence in a similar manner as the phosphorylated animal bacterial effectors. The identification and characterization of the host kinase(s) phosphorylating the CTD (Avk) is needed to elucidate the molecular basic of its interaction with AvrPto, whether or not it is the CTD virulence target, and its function when AvrPto is not present.

I have shown in Chapter 4 that Avk activity is induced by PAMPs and this induction is likely to be suppressed by T3SS effectors, indicating that Avk(s) could be involved in PTI. It is also possible that multiple host kinases phosphorylate AvrPto. Many approaches have been taken in our lab to identify Avk(s), including yeast two hybrid screens with AvrPto CTD variants, biochemical purification, and screening of

candidate kinases. One of our hypotheses is that Avk physically interacts with AvrPto. This hypothesis is supported by the preliminary data from a biochemical purification experiment using an AvrPto affinity column. There it was found that Avk activity could be enriched by using AvrPto but not a C-terminal deletion form of the effector (I. Yeam and G. B. Martin, unpublished data).

Since the binding of a kinase to its substrate is generally weak and transient, some modifications were made to AvrPto prior to performing the Y2H screen in order to enhance the probability of identifying Avk (Manning and Cantley, 2002). AvrPto with Ala substitution at S147 and S149 has been used as the bait to test the 'substrate trap' hypothesis, in which the kinase cannot phosphorylate the CTD and thereby does not release AvrPto (I. Yeam, H. McLane, and G. B. Martin, unpublished data). A PAMP-induced tomato cDNA library was used in this screen. A few potential kinases have been identified to interact with this AvrPto variant; however, whether or not they are Avk remains to be tested.

In Chapter 4, I describe my identification of calcium-dependent protein kinases (CDPKs) as potential Avk's. These were identified by screening candidate kinases in *in vitro* kinase assays using either AvrPto-WT or AvrPto-2XA (Ala substitutions of S147 and S149) as substrates to look for kinases that phosphorylate AvrPto in a CTD (S147 and S149)-specific manner. The candidate kinases were identified from functional protein microarray experiments. In the first experiment, protein microarrays were subjected to *in vitro* autophosphorylation assays either with or without AvrPto. I looked for protein kinases on the protein chips that show altered autophosphorylation level in the presence of AvrPto. This approach is based on the observation that kinase autophosphorylation level is often reduced in the presence of the proper substrate in *in vitro* kinase assay. In addition, I also included in the screen the kinases that bind to AvrPto on the protein microarray as mentioned earlier. The two 'positive' kinases

identified, AtCPK6 and AtCPK30, have reduced autophosphorylation signals when AvrPto is present in the protein microarray experiment as well as in *in vitro* kinase assays. Therefore, this approach could be useful in screens for potential kinases of a substrate of interest. Further investigation of the CDPK kinase family revealed that only CDPKs belonging to clades 1 and 3 but not clades 2 and 4 could phosphorylate the AvrPto CTD *in vitro*, indicating that there is substrate specificity within the family (Cheng et al., 2002).

A tomato CDPK also phosphorylates the CTD, which further supports a role of CDPKs as Avks since Avk activity is conserved in many plant species (Anderson et al., 2006). Although I have not shown that CDPKs are the *in planta* Avk due to functional redundancy and the lack of a specific inhibitor, I did show that Avk activity from plant extracts is Ca^{2+}-dependent, which supports the involvement of CDPKs in this activity. The identification of CDPKs as potential Avks does not exclude the possibility that there are other kinase(s) that might phosphorylate AvrPto besides CDPKs. Candidate kinases from parallel screens in our lab will be subjected to the same screen as the one used to identify CDPKs to find additional candidate Avk(s).

During the characterization of AvrPto phosphorylation, I found a discrepancy between AvrPto phosphorylation specificity between *in vitro* and *in vivo* kinase assays (Chapter 4). It seems that other Ser/Thr residues outside of the CTD are also phosphorylated strongly *in vivo* while only Ser residues within the CTD have been shown to be the major *in vitro* phosphorylation sites. While screening for Avk using purified protein kinases, I also found that AvrPto could be a substrate for certain kinases in a CTD-independent manner (Chapter 4). These kinases might be responsible for phosphorylating non-CTD Ser/Thr sites of AvrPto *in vivo*. However, we showed in our previous studies that the CD loop, which mediates AvrPto binding to the host PRRs, and the phosphorylated CTD appear to account for all the virulence

activity observable with our current assays. It seems unlikely than that these additional *in vivo* phosphorylation events play a significant role in AvrPto virulence.

At the time of writing this dissertation, I do not have conclusive evidence to show whether or not the CTD manipulates CDPKs for its virulence functions. An initial observation that CDPK kinase activity is reduced in the presence of AvrPto suggests that AvrPto may interfere with CDPK activity by mimicking a CDPK substrate(s). However, this hypothesis needs to be tested in further experiments. Previous studies on CDPK provide a range of molecular markers for CDPK downstream signaling pathways (Sheen, 1996; Boudsocq et al., 2010) and these markers will be useful for the elucidation of the effects of the CTD on known CDPK-related responses.

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